Patterns of activity in marine planktonic copepods: diel rhythms and driving factors

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Ph. D. Thesis 2020

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Institut de Ciències del Mar (ICM-CSIC)

Thesis presented to obtain the Doctoral Degree by the University of Barcelona (UB),
Ph. D. program in Marine Sciences

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Preface

The present Ph. D. thesis was conducted in 2016-2020 at Institut de Ciències del Mar (ICM-CSIC) under the supervision of Dr. Enric Saiz Sendrós and Dr. Albert Calbet Fabregat within the frame of the projects FERMI (CGL2014-59227-R) and ZOOTHERM (CTM2017-84288-R). Some experiments were carried out at Kristineberg Marine Research Station (Sweden) in summer 2018 and autumn 2019 during two research stays funded by ASSEMBLE Plus. The Ph. D. student Manuel Olivares was supported financially by an FPU grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

The front and back covers were designed by Manuel Olivares with the help of Claudia Traboni, Aleix Obiol and Adrià Auladell. Both covers are a token of gratitude for all those people who accompanied me during these four years.

Acknowledgements

La elaboración de esta tesis doctoral no habría sido posible sin el apoyo incondicional de las personas que me han acompañado durante estos cuatro años de aventuras y desventuras. Por ello, me gustaría mostrar mi agradecimiento a:

· Enric y Albert, por vuestra excelente supervisión durante la tesis doctoral. Vuestros útiles consejos y entera dedicación han sido fundamentales para mi crecimiento no sólo profesional, sino también personal.

· Mario, por tu compañía y apoyo incondicional en todos los momentos que lo he necesitado.

· Mis padres, por vuestro interés en saber cómo van los experimentos con plancton y alabar mi tarea científica, además de hacerme las cosas más fáciles siempre que habéis podido.

· Mis hermanas, por vuestra alegría contagiosa y vuestros ánimos que, sin duda, han ayudado enormemente a mantener una actitud positiva.

· El resto de mi familia, por vuestra plena disposición a ayudar y a aconsejar en todo momento.

· Mis compañer@s de batalla en el laboratorio Kaiene, Anna, Claudia, Gui, Deju y Queralt. Nuestros momentos juntos han sido fundamentales para mantener los ánimos cuando las cosas no salían como uno esperaba.

· El resto de amig@s del ICM Adrià, Ainara, Aleix, Ana Trindade, Ana Sotomayor, Anna Olivé, Andreu, Ari italiana, Ari leona, Carlos, Carlota, Charlie, Dani, Elena, Guillaume, Guillem, Ina, Isa, Janire, Joan, María, Marina Biel, Marina Pastor, Marta, Miguel, Noe, Patri, Stefano.

· Mi tutora Montse, por confiar en mí y acompañarme con entusiasmo en mis comienzos como docente en la universidad.

· Peter, por toda la ayuda y la atención prestadas durante mis estancias en Kristineberg.

· Mis amig@s Cris, Hodei, Javi, Gemma, Jose Carlos, Manu, Marta, Mike, Isma, Carlos, Irene, Marcos, Celia, Ana, Miriam.

· Las grandes personas que he descubierto y re-descubierto estos cuatro años: Julia, Marina, Isa, Paola, Víctor, Manu, Kirti, Sune, Ola, Alicia, Clara, Marta.

· El personal técnico, de administración y de limpieza del ICM. Nuestra labor no sería posible sin vosotr@s.

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Abstract

Marine planktonic copepods play a key ecological role in pelagic food webs. The study of their patterns of activity is fundamental in order to better understand the processes involved in the transfer of energy from lower trophic levels to higher-level consumers in marine ecosystems. This thesis is an attempt to deepen the knowledge of the factors that affect the activity patterns of marine copepods. Some of these aspects had not been addressed previously and others still required further investigation. In particular, this thesis primarily focuses on the study of daily and diel patterns of feeding of marine planktonic copepods, and the influence of factors like ontogeny, gender, food availability, predation threat, light conditions, mutigenerational rearing in the laboratory, and temperature. The experimental work carried out in this thesis mostly consisted of laboratory incubations using wild and laboratory-reared specimens of the calanoid copepods *Centropages typicus* and *Paracartia grani*. Among the main findings in this thesis are the stage- and gender-specific differences in the feeding patterns of marine planktonic copepods. Moreover, we analyzed the role of predation risk and that of other factors in the modulation of feeding rhythms, and also evaluated the physiological costs related to temperature fluctuations involved in diel vertical migration. The new insights obtained in this thesis will certainly increase our capability to estimate the grazing impact of copepod populations in plankton communities, and will allow us to obtain better estimates of energy transfer in marine pelagic food webs.

Resumen

Los copépodos planctónicos desempeñan un papel ecológico clave en las redes tróficas pelágicas marinas. El estudio de sus patrones de actividad es fundamental para comprender mejor los procesos involucrados en la transferencia de energía desde los niveles tróficos inferiores a los consumidores superiores en los ecosistemas marinos. Esta tesis es un intento de profundizar en el conocimiento sobre los factores que modulan los patrones de actividad de los copépodos marinos. Algunos de estos aspectos no se habían estudiado previamente y otros aún requerían más investigación. En particular, esta tesis se centra principalmente en el estudio de los patrones diarios de alimentación de los copépodos planctónicos marinos y la influencia de factores como la ontogenia, el género, la disponibilidad de alimento, la amenaza de depredación, las condiciones de luz, la crianza multigeneracional en el laboratorio y la temperatura. El trabajo experimental llevado a cabo en esta tesis ha consistido mayoritariamente en incubaciones de laboratorio utilizando especímenes salvajes y de laboratorio de los copépodos calanoideos *Centropages typicus* y *Paracartia grani*. Entre los principales hallazgos en esta tesis destacan las diferencias en los patrones de alimentación entre estadios de desarrollo y entre sexos de copépodos planctónicos marinos. También se analiza el papel del riesgo de depredación y el de otros factores en la modulación de los ritmos de alimentación y se evalúan los costes fisiológicos relacionados con las fluctuaciones de temperatura implicadas en la migración vertical diaria. Los nuevos conocimientos obtenidos en esta tesis aumentarán nuestra capacidad para estimar el impacto de la depredación de las poblaciones de copépodos en las comunidades de plancton y nos permitirán obtener mejores estimaciones de la transferencia energética en las redes tróficas pelágicas marinas.

Resum

Els copèpodes planctònics tenen un paper ecològic clau en les xarxes tròfiques pelàgiques marines. L'estudi dels seus patrons d'activitat és fonamental per a comprendre millor els processos involucrats en la transferència d'energia des dels nivells tròfics inferiors cap als consumidors superiors en els ecosistemes marins. Aquesta tesi és un intent d'aprofundir en el coneixement sobre els factors que modulen els patrons d'activitat dels copèpodes marins. Alguns d'aquests aspectes no s'havien estudiat prèviament i altres encara requerien més investigació. En particular, aquesta tesi se centra principalment en l'estudi dels patrons diaris d'alimentació dels copèpodes planctònics marins i la influència de factors com l'ontogènia, el gènere, la disponibilitat d'aliment, l'amenaça de depredació, les condicions de llum, la criança multigeneracional al laboratori i la temperatura. El treball experimental dut a terme en aquesta tesi ha consistit majoritàriament en incubacions de laboratori utilitzant espècimens salvatges i de laboratori dels copèpodes calanoids *Centropages typicus* i *Paracartia grani*. Entre les principals troballes en aquesta tesi destaquen les diferències en els patrons d'alimentació entre estadis de desenvolupament i entre sexes de copèpodes planctònics marins. També s'analitza el paper del risc de depredació i el d'altres factors en la modulació dels ritmes d'alimentació i s'avaluen els costos fisiològics relacionats amb les fluctuacions de temperatura implicades en la migració vertical diària. Els nous coneixements obtinguts en aquesta tesi augmentaran la nostra capacitat per estimar l'impacte de la depredació de les poblacions de copèpodes en les comunitats de plàncton i ens permetran obtenir millors estimes de la transferència energètica en les xarxes tròfiques pelàgiques marines.

1. General introduction

1.1. Zooplankton: a general overview

The term “zooplankton” refers to the heterotrophic organisms that live drifting in the water, from small protozoans of some micrometres (protozooplankton) to animals (metazooplankton) that may reach sizes from one millimetre or less (e.g. copepods) to a few meters (e.g. some jellyfish) (Fig. 1). These organisms belong to plankton because they do not conduct large-scale displacements against water currents, either because of their small size or their limited swimming activity. Among the metazooplankton, there are animals that spend their whole life in the plankton, such as cladocerans, copepods, or krill, and others that are components of the plankton only during their early life stages, such as the larvae of many molluscs, echinoderms, and fishes. Hence, zooplankton communities usually consist of highly diverse assemblages of species with a wide variety of forms, sizes, and trophic roles (Fig. 1).

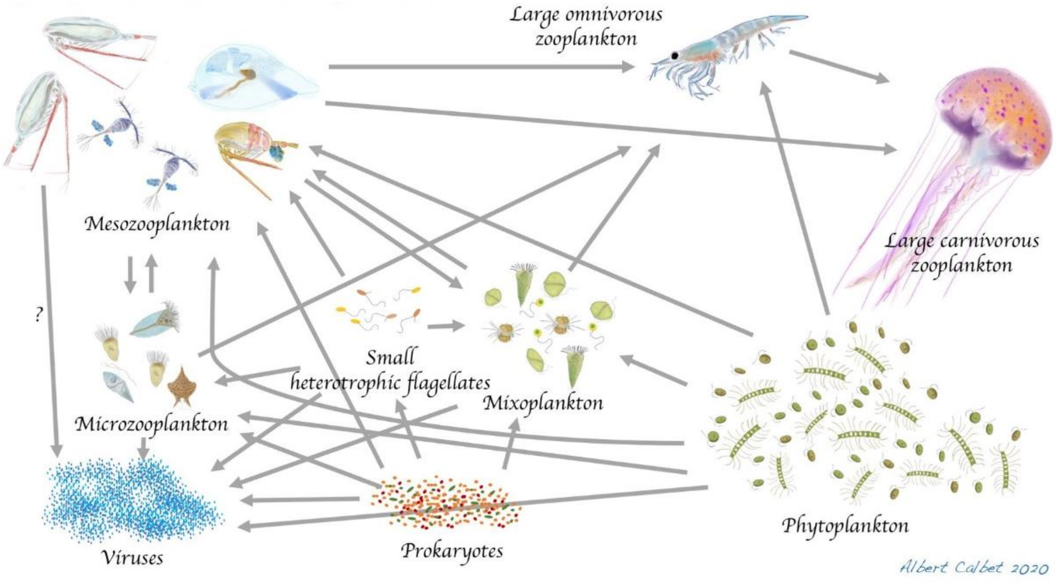

Fig. 1. Marine planktonic food web showing the high diversity of forms and trophic roles within plankton communities. The major trophic interactions among groups are indicated. Figure by Albert Calbet.

Zooplankton are a vital trophic link between primary producers (i.e. phytoplankton) and larger animals (e.g. fish, whales) in pelagic food webs (Runge

1988, Turner 2004) (Fig. 2). Protozooplankton (e.g. ciliates, heterotrophic flagellates) are considered the major grazers of phytoplankton in marine systems (Calbet & Landry 2004, Schmoker et al. 2013) and, unlike most metazoan zooplankton, are capable to feed on the tiny plankton (e.g. bacteria, small phytoplankton). Moreover, some protozoan grazers can perform photosynthesis and act as primary producers (mixoplankton). Among the metazoan components of zooplankton, copepods dominate the marine communities from tropical to polar regions. Their numerical dominance across seas and oceans is so flagrant that these small crustaceans have been pointed out as the most abundant metazoans on Earth (Humes 1994). Copepods typically represent 55-95% of the total abundance, and 25-80% of the biomass of the net zooplankton (Longhurst 1985, Atkinson et al. 1996, Steinberg et al. 2008). In certain geographical locations, copepods can even make up to 96-98% of the biomass (Ward & Shreeve 1999). Planktonic copepods act as important consumers of both phytoplankton and protozooplankton, and represent a fundamental prey item for larger zooplankton (e.g. krill) and other animals like fish (Runge 1988, Atkinson 1996, Turner 2004, Calbet & Saiz 2005) (Fig. 1, 2). Besides copepods, other relevant groups in the metazooplankton include crustaceans like krill, amphipods and decapod larvae, and non-crustacean organisms like salps, doliolids, larvaceans, and chaetognaths. The feeding habits of metazoan zooplankton are highly diverse, going from herbivory through omnivory to carnivory, occasional feeding on detritus and faecal material, and even parasitism of other organisms.

Zooplankton communities can contribute significantly to the regeneration of nutrients within the euphotic layers through excretion (Alcaraz et al. 2010). Moreover, zooplankton contribute to the biological pump (carbon export from surface to depth through biological processes) either through the production of sinking material (e.g. faecal pellets, carcasses, moults), or by the active process of vertical migration (Longhurst et al. 1990, Le Borgne & Rodier 1997, Steinberg et al. 2000) (Fig. 2). Hence, the ecological role of zooplankton is fundamental for the structure and functioning of pelagic ecosystems.

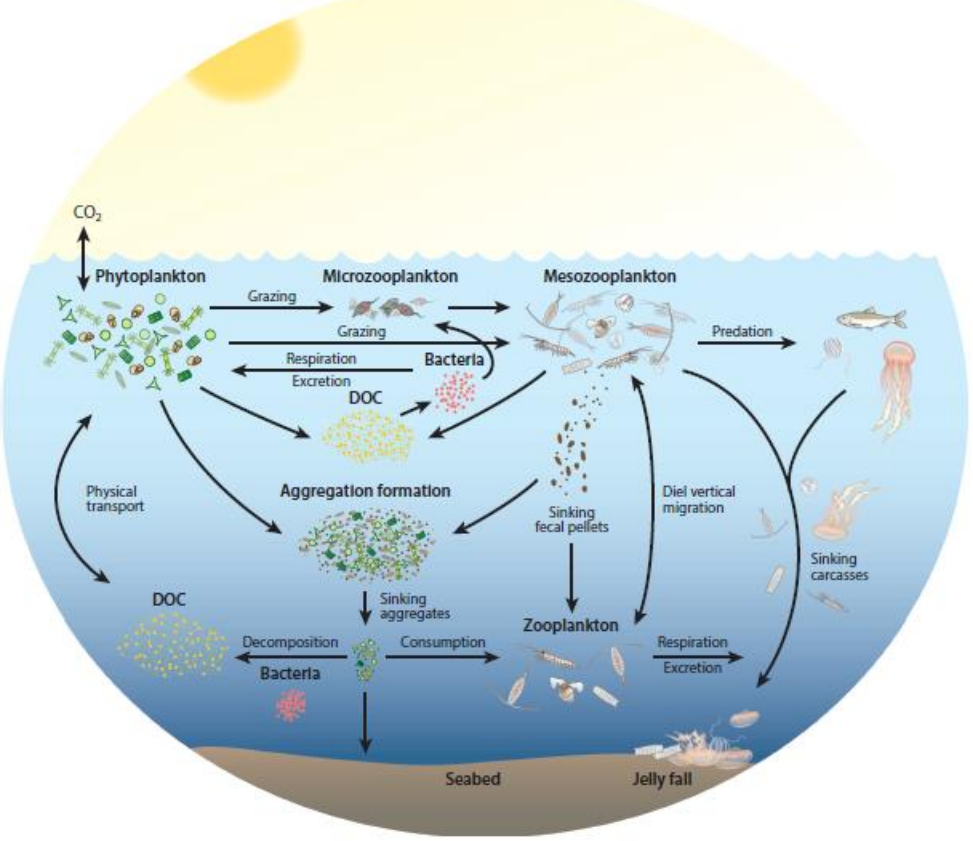

Fig. 2. The biological pump in the ocean. Zooplankton feed on small plankton (e.g. bacteria, phytoplankton) and other zooplankton in euphotic layers, and contribute to the vertical export of carbon to deeper layers through sinking material (faecal pellets, carcasses, moults), and the active process of vertical migration. Extracted from Steinberg & Landry (2017).

1.2. Characteristics of marine planktonic copepods

a) Taxonomy and diversity

Copepods (subclass Copepoda) are small crustaceans belonging to phylum Arthropoda, subphylum Crustacea, class Hexanauplia (WoRMS Editorial Board 2020). All the copepods are aquatic and can be found in marine, brackish and freshwater environments. There are approximately 13000 species of copepods described, among which ca. 2800 species live in fresh waters (Boxshall & Defaye 2008). Their life style is very diverse, from free to parasitic, from planktonic to benthic forms (Boxshall & Defaye 2008). Marine planktonic copepods with a free (non-parasitic) mode of life account for up to 2692 species belonging to 342 genera, 74 families, and 9 orders (Razouls et al. 2020). Among the different orders

(Calanoida, Platycopioida, Misophrioida, Mormonilloida, Cyclopoida, Siphonostomatoida, Harpacticoida, Monstrilloida, Thaumatopsylloida), Calanoida is the richest with about 1800 species (Mauchline 1998).

b) Morphology

Marine planktonic copepods may reach adult sizes that range between 0.1 and 17 mm depending on the species, sex, and habitat (Hirst & Kiørboe 2014, Brun et al. 2017), although most species show typical adult body lengths of 0.5-2 mm (Mauchline 1998). Like other arthropods, copepods have an external skeleton (exoskeleton), a segmented body, and pairs of jointed appendages (Fig. 3). Some body segments might be externally fused and, therefore, the external and internal segmentations might differ (Mauchline 1998). The body can be divided into an anterior part (prosome) and a posterior part (urosome). The prosome is usually broader than the urosome, and contains a single eye, the mouth, two pairs of antennae (the first pair being longer than the second one), several paired mouthparts (mandibles, maxillae, maxillipeds), and 4-5 pairs of swimming legs (Fig. 3). The first antennae are mainly used for sensory perception and mating, the second antennae and the mouthparts are involved in feeding and locomotion, and the swimming legs are mostly used for locomotion and copulation (Gill & Crisp 1985, Paffenhöfer 1998, Van Duren & Videler 2003). The urosome has several segments with no appendages, including a sexual segment for reproduction and an anal segment for defecation (Fig. 3).

c) Life cycle

Planktonic copepods go through a very complex development process from eggs to adults, which normally includes 6 larval (naupliar) stages, and 6 copepodite stages, the last copepodite being the sexually-active adult form (Gilbert & Williamson 1983) (Fig. 4). Moving from one development stage to the next implies severe morphological transformations, and the moult of the exoskeleton to allow the corresponding increase in size and weight (Vidal 1980a). Nauplii have a piriform body with no apparent segmentation, and contains only the first three pairs of appendages (first and second antennae, and mandibles) (Fig. 4). The body of copepodites is more elongated and already shows a clear distinction between the prosome and the urosome (Fig. 4). New segments and legs are added through the different copepodite stages until getting the final adult morphology. Adults frequently show sexual dimorphism (Gilbert & Williamson 1983). The most prominent sexual differences are the body size (females are usually larger), and the morphological modifications that males have in their antennae and fifth legs for copulation (Gilbert & Williamson 1983, Ohtsuka & Huys 2001, Hirst & Kiørboe 2014).

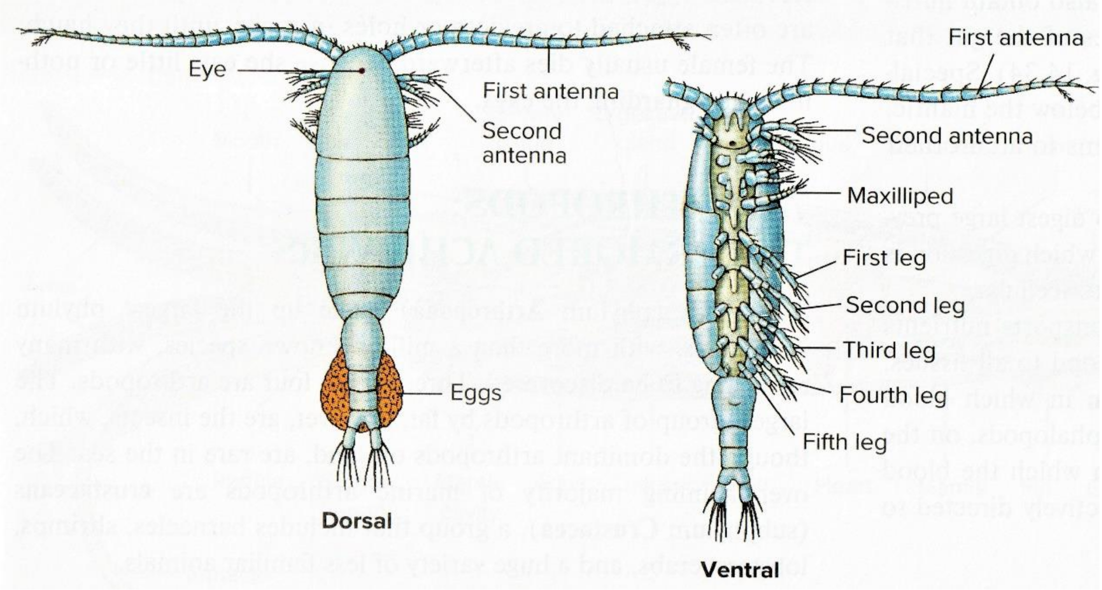

Fig. 3. Dorsal and ventral views of a planktonic copepod. The body is segmented and the anterior part (prosome) is wider than the posterior part (urosome). The prosome contains a single eye, the mouth, and several pairs of appendages with different functions (antennae, mouthparts, legs). The urosome has no appendages. Extracted from Castro & Huber (2019).

d) *Sensory perception*

Planktonic copepods can use their photo-, mechano-, and chemosensory systems to perceive signals from the environment. They have two sensory organs in the head involved in photoreception: the nauplius eye and the Gicklhorn's organ (Elofsson 1970, Frase & Richter 2020). Planktonic copepods may follow light cues to alter their vertical distribution through the water column and avoid predators generating shadows (Stearns & Forward 1984, Buskey et al. 1986). The mechanoreceptors and chemoreceptors are mainly located along the first antennae

(Strickler & Bal 1973, Gill 1986, Bundy & Paffenhöfer 1993, Hartline et al. 1996), which can act as pure mechanoreceptors, pure chemoreceptors, or receptors with a dual function (Weatherby et al. 1994, Lenz et al. 1996, Uttieri et al. 2008). Further mechano- and chemoreceptors can be also found in the mouthparts, the integumental organs along the body, and probably the frontal filaments (Elofsson 1971, Friedman & Strickler 1975, Nishida 1989, Paffenhöfer & Loyd 2000). Planktonic copepods largely rely on mechanoreception and chemoreception to locate and select prey, find mates, and avoid predators, among other purposes (van Duren & Videler 1996, Kiørboe 2013, Heuschele & Selander 2014). The sensitivity of copepods to perceive environmental cues can differ among species, sex and life stages (Yen et al. 1992, Fields & Yen 1997, Kiørboe et al. 1999, Ohtsuka & Huys 2001). For instance, copepodites and adults can respond to lower fluid deformation rates than earlier stages and, therefore, can detect predators at larger distances (Kiørboe et al. 1999, Bradley et al. 2013). Also, males may have their chemosensory system enhanced to detect the pheromones released by females (Boxshall & Huys 1998, Ohtsuka & Huys 2001).

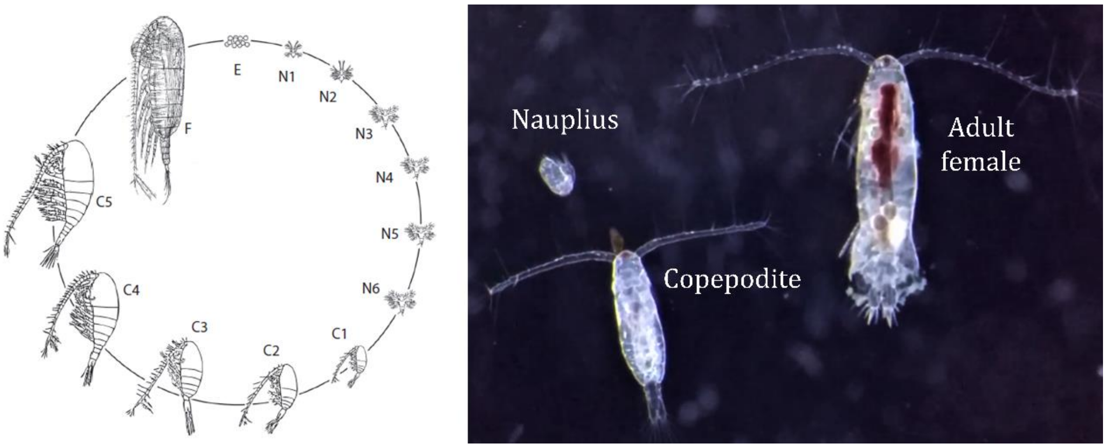

Fig. 4. Life cycle of a planktonic copepod. From eggs (E) to adult females (F), planktonic copepods go through multiple larval (nauplii, N1-6) and juvenile (copepodites, C1-C5) stages. Figure on the left extracted from Baumgartner & Tarrant (2017). Photo on the right (Paracartia grani) by Marine Zooplankton Ecology Group (ICM).

e) Behaviour

Planktonic copepods can be herbivores, omnivores, or carnivores with varying plasticity (Paffenhöfer 1988, Kleppel 1993, Saiz & Calbet 2011). They may also feed on detritus and bacteria (Boak & Goulder 1983, Roman 1984). According to their foraging strategy, planktonic copepods could be classified as ambush feeders (copepods that remain still or passively sinking, and wait for motile prey to attack), feeding-current feeders (copepods that produce feeding currents with their appendages to capture prey), and cruise feeders (copepods that swim actively to encounter prey) (Kiørboe 2011). Their feeding strategy largely determines their swimming behaviour, alternating periods of no motion, sinking, jumps, or active swimming (Tiselius & Jonsson 1990). Factors such as sex, life stage, food conditions, and predation risk may also affect the swimming patterns of planktonic copepods (Van Duren & Videler 1995, Paffenhöfer et al. 1996, van Duren & Videler 1996, Tiselius et al. 1997). For instance, males generally show a more active swimming activity in order to find mates, often guided by pheromone trails released by females (Uchima & Murano 1988, van Duren & Videler 1996, Weissburg et al. 1998, Bagøien & Kiørboe 2005). After mate encounters, males may use their first antennae to grasp females and their modified fifth legs for copulation (Blades 1977, Uchima & Murano 1988, Buskey 1998). Depending on the species, fertilized females may carry their eggs or release them to the environment (Kiørboe & Sabatini 1994).

1.3. Diel activity rhythms in marine planktonic copepods

Marine planktonic copepods are known to show day-night differences in their patterns of activity (i.e. diel rhythms). Among the diel activity rhythms, the diel patterns of vertical distribution along the water column (i.e. diel vertical migration) are the best studied, followed by the diel patterns of feeding.

a) Diel vertical migration

Many copepod species conduct vertical displacements through the water column on a daily basis, typically staying in upper waters at night and moving to deeper, darker layers during the daytime (Roe 1984, Dagg et al. 1989, Peterson et al. 1990, Atkinson et al. 1992) (Fig. 5). The distances covered in vertical migrations are highly variable among species and life stages. Generally, the amplitudes of migration increase from small to larger stages/sizes (Huntley & Brooks 1982, Atkinson et al. 1992). In some cases, vertical migrations imply movements of up to several hundreds of meters through the water column (Wiebe et al. 1992, Al-Mutairi & Landry 2001). This migratory phenomenon plays a fundamental ecological role in the active export of nutrients from shallow to deeper layers by copepods and other zooplankton (Longhurst et al. 1990, Le Borgne & Rodier 1997, Steinberg et al. 2000).

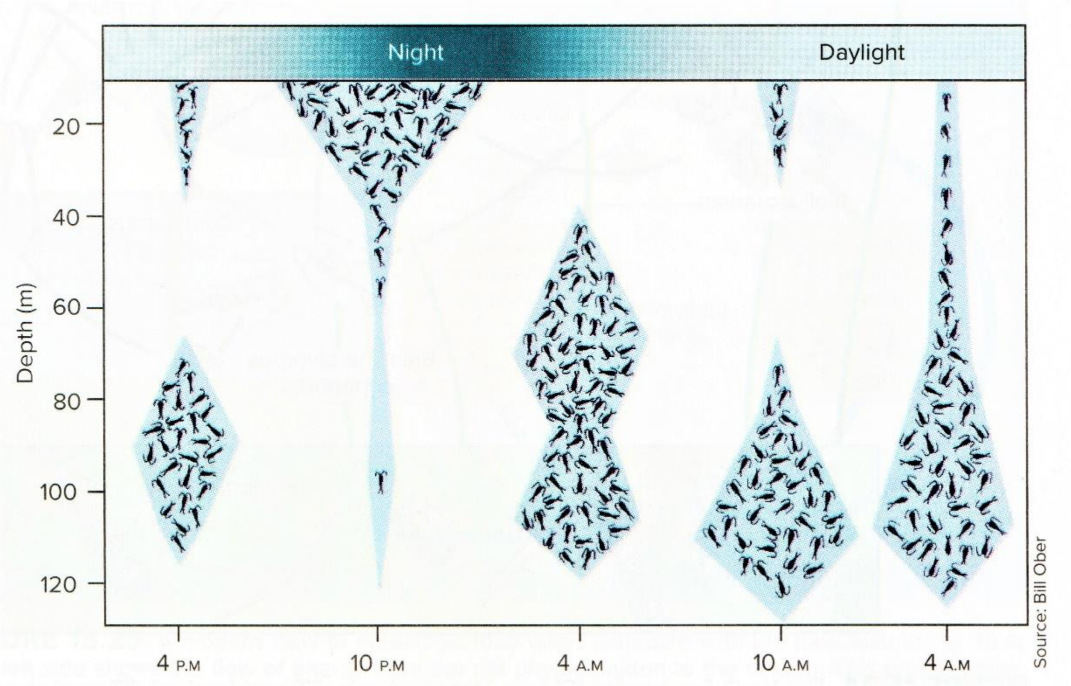

Fig. 5. Diel vertical migration of copepods. Migrating copepods typically remain deeper during the daytime and ascend to upper layers at night. Extracted from Castro & Huber (2019).

The adaptive value of diel vertical migration has been extensively discussed in the past. One of the main drivers that seems to trigger this migratory behaviour is the avoidance of visual predators during daylight hours (Bollens & Frost 1989a, 1991, Neill 1990, Frost & Bollens 1992). In this respect, migrant copepods may show different migration patterns depending on the abundance of predators (Bollens & Frost 1989a, Frost & Bollens 1992, Hays et al. 1996), the type of predator (Ohman 1990, Ghan et al. 1998), the predator cue (Bollens & Frost 1989b), and the size-and stage-specific vulnerability to predation (Neill 1992, Ohman & Romagnan 2016). Despite the adaptive benefits of vertical migration (e.g. avoidance of predators), migrant zooplankton may face important changes in environmental conditions through the water column (e.g. food, temperature) that normally imply metabolic disadvantages compared to non-migrating conditions in food-enriched upper, warmer layers (Orcutt & Porter 1983, Stich & Lampert 1984, Frost 1988, Lampert et al. 1988, Loose & Dawidowicz 1994). Therefore, the diel migration patterns of planktonic copepods fundamentally reflect a trade-off between eating and growing on the one hand, and not being eaten on the other hand.

b) Diel feeding rhythms

Marine planktonic copepods frequently show higher feeding activity at night (Fig. 6). In some cases, the copepod feeding rhythms are a consequence of nocturnal forays into food-rich upper layers during diel vertical migration (Gauld 1953, Dagg et al. 1989, 1998, Atkinson et al. 1992, Tang et al. 1994). However, planktonic copepods may show diel rhythms of feeding in the absence of vertical migration (Hayward 1980, Head et al. 1985, Durbin et al. 1995), and even at low food concentrations (Durbin et al. 1990, Calbet et al. 1999). Therefore, copepod feeding rhythms seem to have an adaptive significance beyond vertical migration, although the factors that trigger and/or modulate them are still poorly investigated.

Wild copepods can show stronger feeding rhythms than those grown in the laboratory for many generations (Calbet et al. 1999). The weakening or absence of feeding rhythms in laboratory-reared copepods have been ultimately attributed to the lack of potential predators in the environment. In this regard, feeding in copepods implies higher motility and conspicuousness, which increases their detectability and vulnerability to predation, particularly during the daylight hours (Tsuda et al. 1998, Torgersen 2001). As it happens with vertical migration, predation risk likely plays an important role in the diel feeding behaviour of planktonic copepods (Bollens & Stearns 1992, Cieri & Stearns 1999), although the effects of predator cues on copepod feeding behaviour still remain questionable (Kiørboe et al. 2018). Considering the potential role of predation threat, it is likely that the diel feeding behaviour of planktonic copepods is highly determined by stage-specific predation vulnerability. However, there are no previous studies that compared the diel feeding patterns of early and late stages of planktonic copepods to confirm or reject this hypothesis. Also, environmental factors such as food availability (Hassett & Blades-Eckelbarger 1995, Calbet et al. 1999) and seasonality (Durbin et al. 1995, Irigoien et al. 1998), or even endogenous components (Stearns 1986, Olsen et al. 2000), seem to affect the intensity of copepod feeding rhythms, but their actual influence has not been fully determined. Thus, further investigation is still required in order to identify the driving factors that trigger and/or modulate the diel feeding rhythms of marine planktonic copepods. This thesis is an attempt to investigate those factors affecting the diel feeding behaviour of marine planktonic copepod (*see Chapters 4.2 and 4.3*).

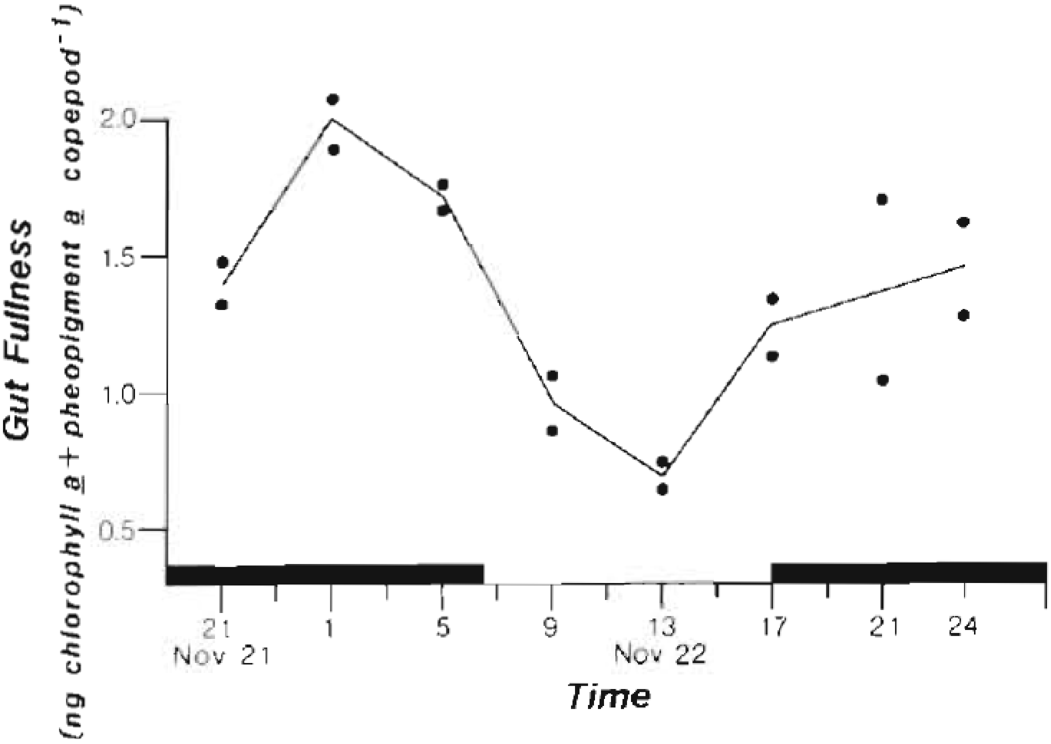

Fig. 6. Feeding activity of a marine planktonic copepod through a day-night cycle. Many copepod species show a higher feeding activity during night hours. Extracted from Stearns (1986).

c) *Other diel rhythms*

Besides migration and feeding, marine planktonic copepods may exhibit diel rhythmicity in other activities that are not directly addressed in this thesis. For instance, planktonic copepods can show important diel variations in their locomotion patterns (Harris 1963, Champalbert 1978) and respiration rates (Cervetto et al. 1993, Pavlova 1994), with peaks of maximum and minimum intensity at different times of the day depending on the species. Also, planktonic copepods usually show a night-skewed egg-laying behaviour, with increasing egg production rates particularly at the end of the night (Stearns et al. 1989, Cervetto et al. 1993, Hopcroft & Roff 1996).

1.4. Driving factors of patterns of activity in marine planktonic copepods

a) *Food availability*

Food availability is a major factor affecting the feeding patterns of planktonic copepods (Frost 1972, Fernández 1979, Kiørboe et al. 1982), which implies indirect effects on other functional traits such as development time, growth rate, egg production, or body size (Vidal 1980a b, Klein Breteler & Gonzalez 1988, Ban 1994, Klein Breteler et al. 1995). Considering the key ecological role of planktonic copepods in pelagic food webs, the study of copepod feeding rates across a range of food concentrations (feeding functional response) is essential knowledge to improve our capability to estimate energy fluxes through secondary production in marine ecosystems (Buitenhuis et al. 2006, Gentleman & Neuheimer 2008, Wollrab & Diehl 2015).

Generally, the ingestion rates of copepods increase with food concentration and become steady above food saturation conditions (Fig. 7). At low food availability copepods may show foraging efforts (e.g. clearance rates) that are higher (Holling Type II response) or lower (Holling Type III response) than at intermediate food concentrations (Holling 1966) (Fig. 7). Copepods with a more passive foraging strategy (e.g. ambush feeders) frequently exhibit Holling Type II responses, whereas other copepods with a feeding mode that requires higher energy demands (e.g. feeding-current feeders or cruise feeders) usually show Holling Type III responses (Kiørboe 2011, Kiørboe et al. 2018).

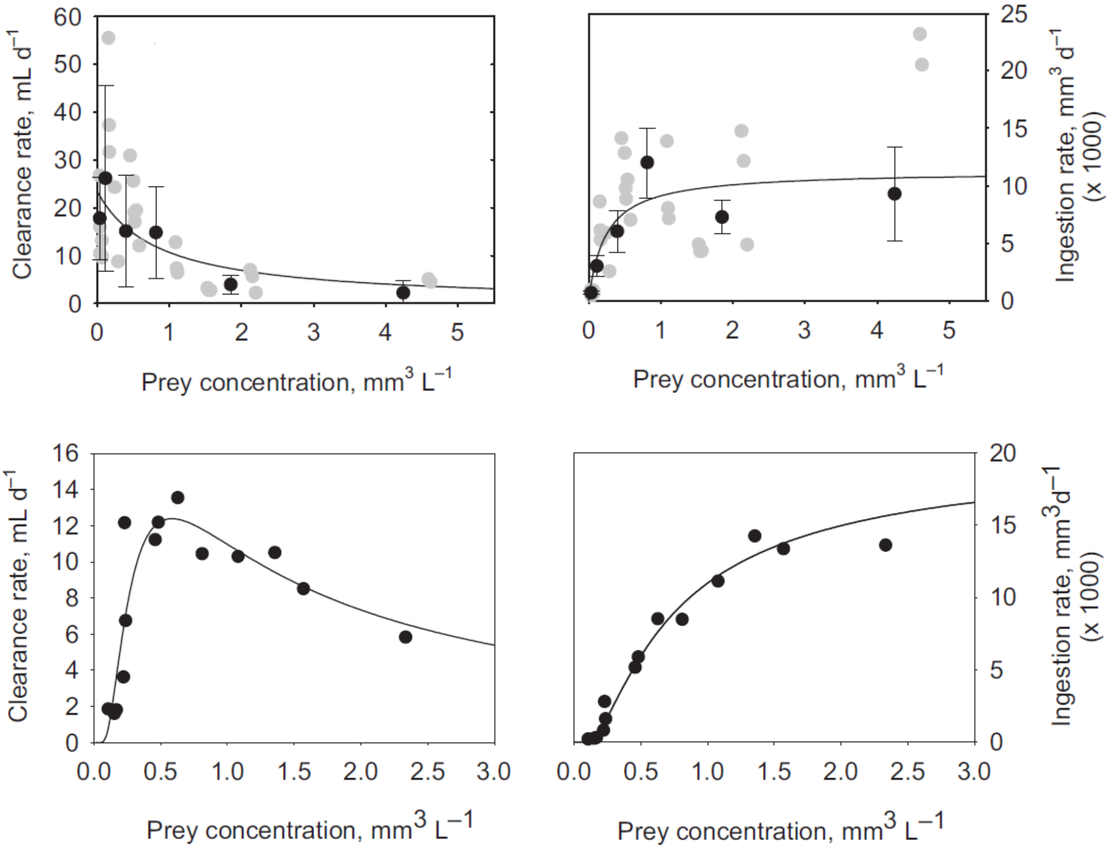

Fig. 7. Clearance and ingestion rates of a marine planktonic copepod across a range of food concentrations (feeding functional response). Above: Holling Type II response. Below: Holling Type III response. Extracted from Kiørboe et al. (2018).

The feeding functional responses of copepods have been determined for a wide variety of copepods and prey, particularly calanoids within the genera *Acartia* (Støttrup & Jensen 1990, Durbin & Durbin 1992, Saiz & Kiørboe 1995) and *Calanus* (Frost 1972, Fernández 1979, Durbin et al. 1995). However, former research that addressed feeding functional responses of planktonic copepods mostly focused on adult females, whereas the reports on early stages and males are much scarcer. This clear unbalance is unfortunate considering the ecological relevance and numerical dominance of early copepod stages in marine plankton communities (Ianora & Buttino 1990, Calbet et al. 2001, Lučić et al. 2003, Turner 2004), and that males may represent up to 50% of the adult populations (Kouwenberg 1993, Kiørboe 2006). Previous attempts to determine ontogenetic changes in the feeding functional responses of marine planktonic copepods either excluded adults (Fernández 1979, Almeda et al. 2010) or copepodites (Saiz et al. 2014). The only complete analysis of feeding functional responses that covered the entire copepod development was conducted with the freshwater species *Cyclops vicinus* (Santer & van den Bosch 1994). The lack of a similar complete record using a marine planktonic copepod species motivated that this thesis included an analysis of the variations in the feeding functional response of marine planktonic copepods through naupliar, copepodite, and adult stages (males and females) across a broad range of prey densities (*see Chapter 4.1*).

Food availability may affect not only the daily feeding patterns of marine planktonic copepods, but also their diel patterns (i.e. feeding rhythms). Unlike the feeding functional responses, there are only a few studies that addressed the effects of food availability on the intensity of copepod feeding rhythms. For instance, Hassett & Blades-Eckelbarger (1995) found that the intensity of feeding rhythms (i.e. day-night differences in feeding activity) became larger at lower food availability. Calbet et al. (1999), however, reported that lower food availability did not affect or led to weaker feeding rhythms. Given this controversy, this thesis also included experiments to provide new insights about how food availability may influence the diel feeding behaviour of marine planktonic copepods (*see Chapter 4.3*).

b) Predation risk

The presence of predators can induce a wide variety of responses in marine planktonic copepods that, besides mortality *per se*, may affect their patterns of activity (non-lethal effects of predators). Among the different predator-induced responses, copepods may alter their vertical distribution through the water column (Bollens & Frost 1989a, Bollens et al. 1994, Cohen & Forward 2005), diminish their feeding activity (Wong 1988, Bollens & Stearns 1992, Saiz et al. 1993, Cieri & Stearns 1999), modify their growth and development rates (Bjaerke et al. 2014), change their swimming behaviour (Wong 1988, van Duren & Videler 1996), or delay their reproduction onset (Heuschele et al. 2014). In some cases, the antipredator behaviour of planktonic copepods can be triggered chemically through the exposure to predator exudates (van Duren & Videler 1996, Cieri & Stearns 1999, Cohen & Forward 2005, Lasley-Rasher & Yen 2012, Bjaerke et al.

2014). However, other studies found no evidence of predator exudate effects on the behaviour of marine copepods (Bollens & Frost 1989a, Bollens et al. 1994, Kiørboe et al. 2018). Therefore, the role of predator chemical cues on copepod performance still remains debatable. In this respect, most former research addressing predator effects largely relied on predator chemical cues as predatory signals, making no possible to detect antipredator responses of copepods that are not chemically triggered. In this thesis, the effects of predators on the activity patterns of marine planktonic copepods are further explored, using both freely-swimming predators and predator exudates (*see Chapters 4.2 and 4.3*).

c) Temperature

There is an extensive bulk of literature about the influence of temperature on functional traits of marine planktonic copepods. Temperature can affect copepod fitness through effects on growth and development rates (Vidal 1980a b, Uye 1988, Huntley & Lopez 1992), body size (Huntley & Lopez 1992, Ban 1994), feeding activity (Fernández 1978, Kiørboe et al. 1982, Dam & Peterson 1988), swimming patterns (Gill & Crisp 1985), reproductive output (Ban 1994, Saiz et al. 1998, Holste & Peck 2006), and respiration rates (Fernández 1978, Vidal 1980c, Hirche 1987, Ikeda et al. 2001). Despite the large number of studies addressing temperature effects on copepod performance, there are only a few that considered effects of fluctuations of temperature (Lock & McLaren 1970, Vuorinen 1987, Zhou & Sun 2017). In this regard, many planktonic copepods are daily exposed to temperature fluctuations through diel vertical migration (Wiebe et al. 1992, Saiz et al. 1999, Al-Mutairi & Landry 2001), and their temperature-dependent performance might not be inferred from the study of average temperature effects according to the mathematical property of non-linear averaging known as "Jensen's inequality" (Ruel & Ayres 1999). This issue is of renewed interest given the increased thermal variability predicted in future climate scenarios, which may be detrimental to copepod performance. Among other goals, this thesis aims to assess experimentally the physiological costs associated to temperature fluctuations experienced during diel vertical migration (*see Chapter 4.4.*).

d) *Other factors*

Besides food availability, predation risk, and temperature, there are other relevant environmental factors that can affect the patterns of activity and fitness of planktonic copepods. Some of them would be food quality (Cowles et al. 1988, Jónasdóttir 1994, Klein Breteler et al. 1999), prey size (Frost 1972, Berggreen et al. 1988), toxin presence (Dutz 1998, Koski et al. 1999), salinity (Lance 1963, Holste & Peck 2006), turbulence (Saiz et al. 1992, Saiz & Kiørboe 1995), or oxygen concentration (Stalder & Marcus 1997, Marcus et al. 2004). As the effects of these factors on copepod performance are not directly investigated in this thesis, further information on them will not be provided here.

1.5. Ecology of the species of study: *Centropages typicus* and *Paracartia grani*

The experiments carried out in this thesis were conducted using wild and laboratory-reared specimens of the calanoid copepods *Centropages typicus* and *Paracartia grani*. These marine planktonic copepods reach adult sizes of about 1 mm, although little variations can be found depending on the geographical location (Halsband-Lenk et al. 2002, Boyer et al. 2013). *C. typicus* is one the best studied species among marine planktonic copepods (Mauchline 1998). This copepod species plays a fundamental ecological role in coastal waters of the Mediterranean Sea, the North Sea, and several areas of the East and North Atlantic Ocean, reaching abundances of up to several hundreds of individuals per cubic meter (Ianora & Buttino 1990, Halsband-Lenk et al. 2002, Bonnet et al. 2007, Mazzocchi et al. 2007). Lough & Kristiansen (2015) reported that *C. typicus* is likely the main prey item for juveniles of cod in coastal environments of the Northwest Atlantic. *P. grani* shows a geographical range that is similar to that of *C. typicus*, being present in neritic waters of the Mediterranean Sea, the North Sea, and the East and North Atlantic (Boyer et al. 2012). However, its distribution is often limited to semi-enclosed water bodies (e.g. lagoons, harbours), where it may also reach abundances of several hundreds of individuals per cubic meter (Rodríguez 1983, Sautour & Castel 1993, Boyer et al. 2012, 2013). The wide geographical distribution, the important ecological role, and the similarities between *P. grani* and other well-studied species (e.g. *Acartia tonsa*) make *C. typicus* and *P. grani* good model species of study.

2. Objectives and underlying hypotheses

Marine planktonic copepods play a fundamental ecological role in pelagic communities. Therefore, the study of their patterns of activity is essential knowledge to better understand the transfer of energy through secondary production in marine ecosystems. This thesis is an attempt to deepen the knowledge of the factors that affect the activity patterns of marine copepods. Thus, the main goals of this thesis can be outlined in the following objectives and underlying hypotheses:

Objective 1. To report and analyse the ontogenetic changes in the daily and diel patterns of feeding of marine planktonic copepods.
Our hypothesis is that the severe morphological and behavioural changes occurring through copepod development may motivate important ontogenetic variations in their patterns of feeding. The Chapter 4.1 of this thesis provides a complete analysis of the ontogenetic changes in the feeding functional response of marine planktonic copepods using naupliar, copepodite and adult (males and female) stages across a broad range of prey concentrations. Additionally, the Chapter 4.2 of this thesis includes the comparison between the diel feeding patterns of nauplii and adults.

Objective 2. To determine the importance of exogenous cues in the diel feeding patterns of marine planktonic copepods.
The relevance of exogenous and/or endogenous components in the diel feeding behaviour of planktonic copepods remains unclear. If exogenous factors are important, we hypothesize that the feeding rhythms of wild copepods would be weakened or loss through the first generations of rearing in the laboratory. However, if copepod rhythms mostly follow endogenous cues, we would expect that the feeding rhythms would be maintained with little changes through multigenerational laboratory rearing. The Chapter 4.2 of this thesis includes the tracking of changes in the diel feeding patterns of marine planktonic copepods from wild specimens through the next generations in the laboratory in order to

provide new insights about the exogenous and/endogenous character of copepod feeding rhythms.

Objective 3. To determine the role of predation risk in the diel feeding behaviour of marine planktonic copepods.

The indirect effects of predation on copepod feeding behaviour are still poorly studied. Feeding in copepods implies higher motility and conspicuousness, which is associated to higher predation risk, particularly during daylight hours. Therefore, we hypothesize that predation risk plays a fundamental role in shaping the diel feeding patterns of planktonic copepods. The Chapters 4.2 and 4.3 of this thesis further explore the importance of predation threat on the diel feeding behaviour of marine planktonic copepods, using wild and laboratory-reared copepods exposed to freely-swimming predators and predator exudates.

Objective 4. To analyse the influence of factors other than predation threat on copepod feeding rhythms.

The diel activity patterns of planktonic copepods might be influenced by environmental factors other than predation threat. We hypothesize that factors such as food availability, which determines the foraging activity of copepods, and seasonal photoperiod, which establishes the timing of day-night cycles, may also modulate the intensity of copepod feeding rhythms. The Chapter 4.3 of this thesis deepens into the influence of seasonal photoperiod (i.e. daytime length) and prey availability on the diel feeding behaviour of planktonic copepods.

Objective 5. To determine the physiological costs of diel temperature fluctuations during vertical migration in marine planktonic copepods.

Many copepod species are daily exposed to diel temperature fluctuations through vertical migration. The temperature-dependent performance of migrant copepods, however, might not be necessarily inferred from average temperature effects. Our hypothesis is that migrant copepods may have physiological mechanisms to compensate temperature fluctuations during vertical migration. The Chapter 4.4 of this thesis deepens into the capability of marine planktonic copepods to cope with diel temperature fluctuations involved in vertical migration, providing new insights about the susceptibility of these animals to the increased thermal variability predicted in future climates.

3. Supervisors' report on the impact factor of published articles

The research conducted within the frame of this thesis led to the publication of three articles (Chapters 4.1, 4.2 and 4.3 of this thesis) and the preparation of a fourth manuscript (Chapter 4.4) that will be submitted to a scientific journal soon. The Ph. D. student Manuel Olivares played a leading role in all the research contributing to the experimental design, the field and laboratory work, the data analysis/interpretation, and the manuscript preparation. The three articles published in the present thesis and the journal impact factors are:

· Olivares, M.; Saiz, E.; Calbet, A. (2019). Ontogenetic changes in the feeding functional response of the marine copepod *Paracartia grani*. Marine Ecology Progress Series, 616, pp. 25-35. https://doi.org/10.3354/meps12928
Research impact. CiteScore 2019: 4.2; SJR 2019: 1.25 (Q1)

· Olivares, M.; Calbet, A.; Saiz, E. (2020). Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms. Aquatic Ecology, 54, pp. 697-709. https://doi.org/10.1007/s10452-020-09768-8
Research impact. CiteScore 2019: 4.8; SJR 2019: 0.77 (Q2)

· Olivares, M.; Tiselius, P.; Calbet, A.; Saiz, E. (2020). Non-lethal effects of the predator *Meganyctiphanes norvegica* and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod *Centropages typicus*. Journal of Plankton Research, 42(6), pp. 742-751.
https://doi.org/10.1093/plankt/fbaa051
Research impact. CiteScore 2019: 3.9; SJR 2019: 0.94 (Q1)

Dr. Enric Saiz Sendrós	**Dr. Albert Calbet Fabregat**
Ph. D. supervisor	Ph. D. supervisor

4. Publications

4.1. Ontogenetic changes in the feeding functional response of the marine copepod *Paracartia grani*

Reference:
Olivares, M.; Saiz, E.; Calbet, A. (2019). Ontogenetic changes in the feeding functional response of the marine copepod *Paracartia grani*. Marine Ecology Progress Series, 616, pp. 25-35. https://doi.org/10.3354/meps12928

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a) Abstract

The feeding activity of copepods is crucial for the structure and functioning of marine ecosystems. Quantification of feeding rates of different copepod life stages across a range of prey densities (functional response) is essential knowledge for improvement of plankton dynamic models. In this study, we conducted experiments to compare the feeding functional responses of nauplii, copepodites, and adults of the marine copepod *Paracartia grani* (formerly *Acartia grani)* when grazing on the flagellate *Rhodomonas salina*. We found that all copepod stages followed a sigmoid curve in their functional responses (Holling Type III model), indicating a metabolic threshold constraining foraging effort at low prey densities. Maximum clearance rates of nauplii and copepodites increased with body mass with a power scaling factor of 1, but maximum clearance rates of adults did not follow the pattern observed for juvenile stages, likely because of the relatively small prey size used in the experiments. Copepod maximum ingestion rates, however, showed allometric scaling along ontogeny that was closer to the typical 0.75 power law and seemed to not be so dependent on prey size. The insights obtained from our study highlight stage-specific differences in copepod feeding activity, and can help improve our capability to estimate the energy flow through copepods in marine food webs.

b) Introduction

Zooplankton constitute the main link between primary producers (phytoplankton) and higher trophic levels in marine food webs (Calbet 2001, Calbet & Saiz 2005). They are a key component of the biological pump and play a major role in nutrient recycling in the photic layer (Steinberg et al. 2000, Alcaraz et al. 2010, Turner 2015). Within marine mesozooplankton, copepods typically account for 35–70% of the biomass and represent more than 90% of the abundance (Longhurst 1985). Consequently, the resource exploitation conducted by copepods is crucial for the structure and functioning of marine ecosystems.

Functional responses of copepods are defined as the relationship between their vital rates (e.g. feeding rates, egg production rates) and prey availability (Solomon 1949, Holling 1966). Because of the major role of copepods in the zooplankton, copepod functional responses have great implications for plankton population dynamics (Runge 1988, Gentleman & Neuheimer 2008, Kiørboe et al. 2018). The quantification of functional responses in both field and laboratory studies enables the development of mathematical models to forecast the response of plankton communities to changing species densities (Morozov 2010, Morozov et al. 2012). These predictive models are intrinsically very sensitive to the parameterization of functional responses (e.g. the shape of the functional response) and for this reason, the proper analysis of copepod functional responses becomes an imperative task to improve our capability to assess potential variations in the future composition of plankton communities and the biogeochemical fluxes that occur through them (Gentleman & Neuheimer 2008, Wollrab & Diehl 2015, Egilmez & Morozov 2016).

Traditionally, the study of feeding activity patterns in copepods has been limited mostly to adults, particularly females. In this respect, feeding functional responses have been well documented for adults of key marine copepod species using a wide variety of prey types, and the records are much more numerous for females than for males (Støttrup & Jensen 1990, Isari & Saiz 2011, Gonçalves et al. 2014, Saiz et al. 2014). Females have been preferred over males in grazing experiments mainly because of their longer life span, higher abundances in natural populations, and fundamental role in recruitment (Kouwenberg 1993, Kiørboe 2006, Kiørboe et al.

2015). However, evidence of distinct feeding behaviours between the sexes (e.g. lower feeding rates in males) demonstrates that gender differences should be taken into account when analysing feeding activity patterns in marine copepods (Lampitt 1978, Uye & Kayano 1994, Saage et al. 2009, Chen et al. 2010, van Someren Gréve et al. 2017b).

Similar to males, juvenile copepods (i.e. nauplii and copepodites) have been greatly neglected in previous research addressing copepod feeding patterns. Juvenile copepods outnumber adults throughout the year in plankton communities (Webber & Roff 1995, Calbet et al. 2001, Turner 2004). They play a key ecological role as major grazers of phytoplankton and small heterotrophs. Therefore, studying their feeding activity is fundamental to evaluating the grazing pressure on the smaller plankton fraction in marine ecosystems (Lučić et al. 2003, Turner 2004, Almeda et al. 2011). The first investigations that focused attention on the feeding patterns of the early stages of copepods focused on their rates of grazing on phytoplankton (Mullin & Brooks 1967, Paffenhöfer 1971, Fernández 1979). In response to increasing interest in this topic, further investigations were carried out on other aspects, such as their foraging behaviour, prey size spectrum, and prey selection (Berggreen et al. 1988, Paffenhöfer 1998, Henriksen et al. 2007, Helenius & Saiz 2017). In terms of feeding functional responses, Fernández (1979) was the first author to provide empirical data on naupliar grazing rates of *Calanus pacificus* across a wide range of prey types and food concentrations. Since then, feeding functional responses have been described for juvenile copepod stages of other genera, such as *Acartia/ Paracartia* (Henriksen et al. 2007, Ismar et al. 2008, Helenius & Saiz 2017), *Oithona* (Henriksen et al. 2007, Almeda et al. 2010, Saiz et al. 2014), and *Centropages* (López et al. 2007).

Despite this valuable information, we think that the current literature on marine copepods has not completely addressed the changes in feeding functional response through the entire copepod life cycle. For instance, Almeda et al. (2010) analysed feeding functional responses up to early copepodites, and Saiz et al. (2014) worked with nauplii and adults but not with copepodites. Berggreen et al. (1988) reported changes in the prey size spectrum of *Acartia tonsa* from nauplii to adults, but in their study, the feeding functional responses were not analysed. Thus, it appears that we still lack a complete record regarding how feeding functional patterns change through ontogeny in marine copepods. To the best of our knowledge, only Santer & van den Bosch (1994) have studied this issue, with the freshwater species *Cyclops vicinus*.

Thus, the main goal of this study was to assess ontogenetic changes in the feeding functional response of marine copepods. To accomplish our objective, we conducted grazing experiments in the laboratory with different development stages of the calanoid copepod *Paracartia grani* (formerly *Acartia grani)* and a broad range of prey concentrations of the flagellate *Rhodomonas salina*. *P. grani* is a common neritic species that has been observed from high to middle latitudes in the eastern Atlantic and across the Mediterranean Sea (Boyer et al. 2012). In our study, we compared feeding functional patterns among different *P. grani* life stages (nauplii, copepodites, adults) and sexes (males and females). We expected that the values of the fundamental parameters defining the feeding functional response (maximum clearance and ingestion rates, half-saturation constants) would be influenced by changes along ontogeny in the allometric scaling of metabolic requirements (Ikeda et al. 2001, Saiz & Calbet 2007) and variations in morphological and behavioural traits (Paffenhöfer 1998).

c) Material and methods

Experimental cultures

For the experiments, the cryptophyte *Rhodomonas salina* was used as prey (equivalent spherical diameter [ESD]: 6.9 µm, carbon [C] content: 38 pg cell^{-1}, nitrogen [N] content: 7.2 pg cell^{-1}; Helenius & Saiz 2017). A culture of *R. salina* was grown in f/2 medium (Guillard 1983) and kept in the exponential growth phase by diluting 1/3 of the culture daily. For the copepods, a cohort of *Paracartia grani* was obtained from a stock culture maintained at the Instituto de Ciencias del Mar (ICM, CSIC) for more than 10 yr (Saiz et al. 2015). To produce the cohort, eggs were siphoned out from the bottom of the copepod stock culture and inoculated into 20 l polycarbonate tanks filled with 0.1 µm filtered seawater. Approximately 24 h after the egg addition, unhatched eggs were removed from the bottom with a siphon to ensure the uniformity of the cohort. The abundance of copepods in the cohort tank started with ca. 15 000 nauplii, and decreased as copepods were taken out for experiments (ca. 1000 adults by the end). The cohort was fed ad libitum at concentrations ranging from 7 (nauplii) to 20 (adults) ppm of *R. salina*. *R. salina* is a nutritionally sufficient prey that has been commonly used in the maintenance of *Acartia* cultures (Støttrup & Jensen 1990, Broglio et al. 2003, Saiz et al. 2015). Both the phytoplankton and the copepod cultures used for the experiments were kept in the laboratory in a cold room at 19 ± 1°C and under a 10 h light:14 h dark photoperiod.

Functional response experiments

In total, 6 feeding functional response experiments were carried out successively using different copepod life stages from the same *P. grani* cohort, encompassing 2 experiments conducted with nauplii, 2 experiments with copepodites, and 2 experiments with adults (one with males and one with females). Prior to each experiment, copepods were collected from the cohort using a 60 μm (nauplii and copepodites) or a 200 μm (adults) mesh and placed in filtered seawater for 2.5–3 h while setting up the experiment. For each functional response experiment, 6 prey concentrations of exponentially growing *R. salina* were prepared. The range of prey concentrations was based on previous knowledge (Calbet & Alcaraz 1997, Helenius & Saiz 2017) to cover limiting to satiating food conditions (500–12 000 cells ml^{-1} for NII– III, 500–14 000 cells ml^{-1} for NV–VI, 500– 16 000 cells ml^{-1} for CI– II, 1000–20 000 cells ml^{-1} for CIII– IV, and 1000–24 000 cells ml^{-1} for adults). Prey concentrations were measured with a Beckman Coulter Multisizer III particle counter. A total of 8 bottles were filled with each prey concentration: 2 served as initial bottles (only prey), 3 as control bottles (only prey), and 3 as experimental bottles (prey and copepods). Bottle volumes ranged from 72 ml for early nauplii to 620 ml for adults. All bottles were filled following a 3-step procedure (one-third of the bottle was sequentially filled each time) to ensure homogeneity of prey densities among bottles. Inorganic nutrients (5 ml l^{-1} of f/2 solution, i.e. a final concentration equivalent to f/400) were also added to each bottle to compensate for any differential effect of copepod nutrient excretion on algal growth. Once the bottles were filled with the prey suspensions, the copepods were added to the bottles either as aliquots (nauplii and copepodites) or individually with a pipette (adults). For the aliquots, the concentration of nauplii/copepodites in the filtered seawater was estimated on the basis of replicated counts of subsamples drawn with an automatic pipette, and then the aliquot volume of the copepod suspension required to reach the desired copepod concentration was added to each experimental bottle. To compensate for dilution in the experimental bottles, the same volume of filtered seawater was added to the initial and control bottles. Copepod adults were transferred to suspensions of the respective prey concentration to avoid any dilution effects, and then they were individually removed and transferred to the experimental bottles. Given that different copepod stages (sizes) and different prey concentrations were tested, the number of copepods added to each bottle was adjusted based on unpublished previous data to result in a reduction of ca. 30% of the prey concentration during incubation. Thus, the number of individuals incubated in the bottles ranged from low to high copepod abundance across the different prey concentrations (80−240 NII– III, 40−160 NV–VI, 40−220 CI– II, 25−90 CIII– IV, 10−35 adult males, and 10−30 adult females). All bottles were sealed with plastic film to avoid air bubbles and capped. The initial bottles were sacrificed at the very beginning of the incubation periods to assess actual initial prey concentrations using the Coulter counter. Some extra copepods were preserved in 4% formaldehyde for measurement of initial copepod size. Control and experimental bottles were mounted on a rotating plankton wheel (0.2 rpm) and incubated for 20−24 h at 19 ± 1°C and under a 10 h light:14 h dark cycle (photosynthetically active radiation [PAR] 15−34 μmol m^{-2} s^{-1}). After the incubations, suspensions from control and experimental bottles were filtered through a submerged sieve with a 60 µm (nauplii and copepodites) or a 200 µm (adults) mesh to separate copepods from prey. The final prey concentrations in each bottle were measured, as they were for the initial bottles, with the Coulter counter. The experimental copepods were checked for activity under a stereomicroscope and then fixed with formaldehyde (4% final concentration) for quantification, stage determination, and final body size measurements. Prey sizes were calculated as the geometric mean between initial and final prey volumes measured with the particle counter. Initial and final copepod sizes were determined by photographing 30−40 nauplii or copepodites and 20−25 adults with an inverted microscope and measuring their body (nauplii) or prosome (copepodites, adults) lengths with ImageJ software (Schneider et al. 2012). Copepod sizes were calculated for each prey concentration as the arithmetic mean between their initial and final mean lengths.

Table 1. Parameters of the feeding functional responses of Paracartia grani on Rhodomonas salina. Maximum clearance rates (F_{max}), maximum ingestion rates (I_{max}), half-saturation constants (K_m), and prey concentrations at which 90% of the maximum ingestion rates were reached (C_{90}) are shown for the different copepod stages and sizes (N: nauplii; C: copepodites; Ad: adults). Means ± SE are provided

Copepod stage	Copepod size (μm)	Fmax (ml ind.$^{-1}$ d^{-1})	Imax (cells ind.$^{-1}$ d^{-1})	Imax (% body C d^{-1})	K_m (cells ml^{-1})	C90 (cells ml^{-1})
NII–III	139 ± 2	0.36 ± 0.02	1623 ± 46	213 ± 6.5	2272 ± 138	6815
NV–VI	238 ± 2	1.29 ± 0.06	5623 ± 185	207 ± 6.5	2188 ± 136	6563
CI–II	457 ± 3	6.25 ± 0.34	21649 ± 659	265 ± 7.6	1733 ± 120	5198
CIII–IV	632 ± 2	15.7 ± 0.98	52761 ± 1290	195 ± 5.4	1684 ± 121	5051
Ad males	922 ± 1	8.98 ± 0.46	67772 ± 1858	68 ± 1.9	3773 ± 243	11318
Ad females	1052 ± 3	15.9 ± 0.57	218457 ± 6401	124 ± 3.3	6877 ± 368	20632

Data analysis

Average prey concentrations and copepod feeding rates (clearance and ingestion rates) were determined according to equations in Frost (1972).

Holling Type III curves were fitted to copepod feeding rates as a function of prey concentration using the software package SigmaPlot v.14.0. Curve fitting was carried out by non-linear regression following the equations in Helenius & Saiz (2017):

$$F = \frac{I_{max}C}{C^2 + {K_m}^2} \tag{1}$$

and

$$I = \frac{I_{max}C^2}{C^2 + {K_m}^2} \tag{2}$$

where F is the clearance rate, I is the ingestion rate, $I_{\max}$ is the maximum ingestion rate, C is the prey concentration, and K_m is the half-saturation constant. Estimates of $I_{\max}$, K_m and prey concentration at which 90% of the maximum ingestion rate was reached (C_{90}) were obtained directly from the Holling Type III model. Maximum clearance rates ($F_{\max}$) were calculated as described in Helenius & Saiz (2017):

$$F_{max} = \frac{I_{max}}{2K_m} \quad (3)$$

Carbon-specific feeding rates were calculated using the conversion factor 0.221 pg C µm^{-3} for *R. salina* (Helenius & Saiz 2017) and the length−weight relationships for *P. grani*: $W = 3.24 \times 10^{-7} L^{2.34}$ for nauplii and $W = 1.12 \times 10^{-10} L^{3.58}$ for later stages (E. Saiz et al. unpubl. data), where W is the copepod mass in µg C, and L is the body (nauplii) or prosome (copepodites and adults) length in µm.

The relationships between copepod body mass and maximum feeding rates (clearance and ingestion rates) were adjusted to a power function. The power fits were obtained from linear regression after log transformation of the variables using the software KaleidaGraph 4.5.4. To improve the robustness of the fitting, for this analysis, we added additional estimates of $I_{\max}$ of *P. grani* obtained from unpublished experiments (M. Olivares et al. unpubl. data) conducted under similar conditions.

d) *Results*

The average size of the copepods used in the functional response experiments ranged from 139 µm in early nauplii to 1052 µm in adult females (Table 1). Mean prey size was similar in all experiments (range 7.1–7.4 µm ESD). All stages of *Paracartia grani* showed a Holling Type III feeding functional response when grazing on *Rhodomonas salina*. Clearance rates peaked at an intermediate prey concentration and decreased at lower and higher concentrations (Fig. 1). $F_{\max}$ increased with stage/size, from 0.36 ml ind.$^{-1}$ d^{-1} for early nauplii to 15.9 ml ind.$^{-1}$ d^{-1} for adult females; the $F_{\max}$ values for adult males were, however, lower than the estimates for late copepodites and females (Table 1). Ingestion rates increased with prey concentration following a sigmoid curve and became steady after reaching food saturation (Fig. 1). I_{max} increased from ca. 1600 cells ind.$^{-1}$ d^{-1} (ca. 0.08 µg C ind.$^{-1}$ d^{-1}) in early nauplii to ca. 218 000 cells ind.$^{-1}$ d^{-1} (ca. 9.12 µg C ind.$^{-1}$ d^{-1}) in adult females (Table 1). The I_{max} values for females were approximately 3 times higher than those of males.

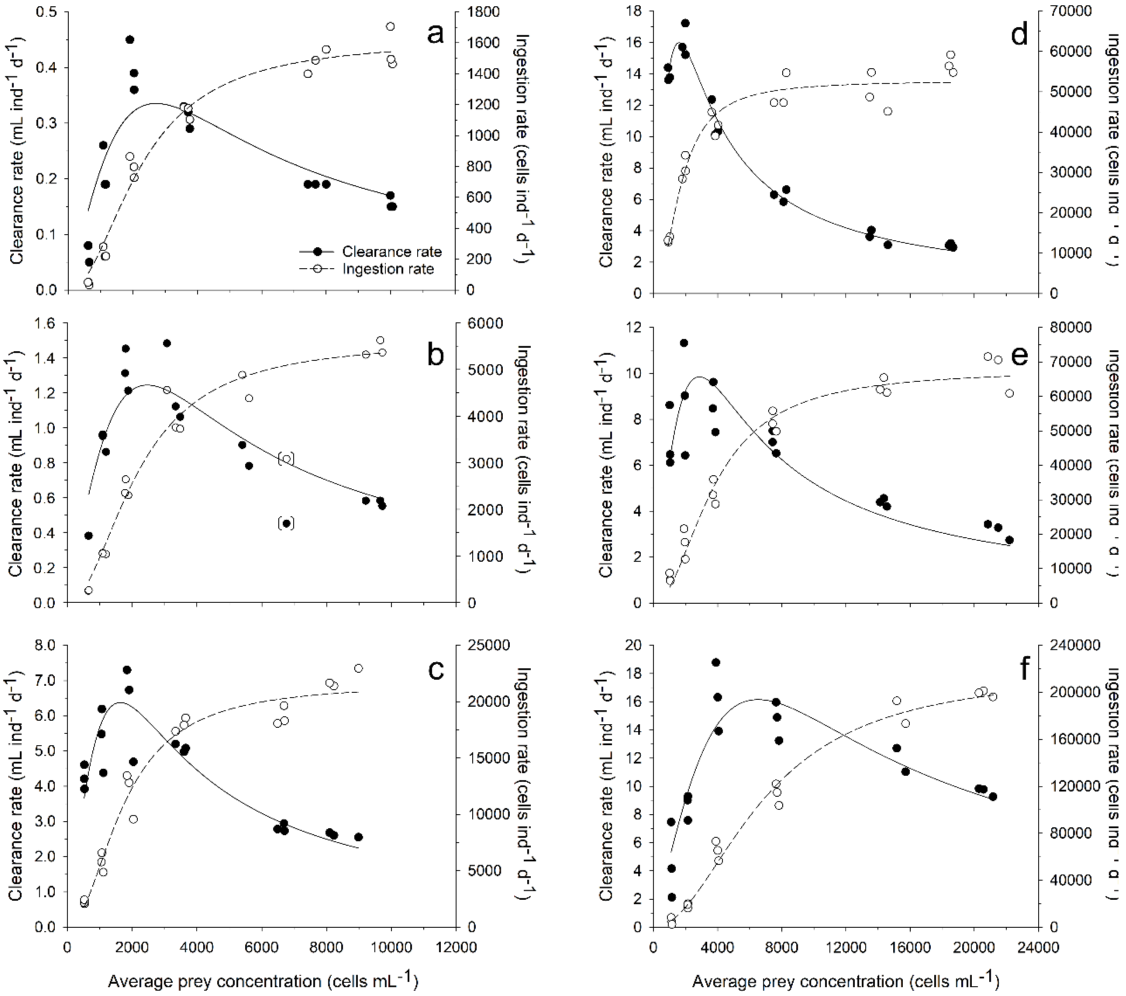

Fig. 1. Functional responses of (a) nauplii II–III, (b) nauplii V–VI, (c) copepodites I–II, (d) copepodites III–IV, (e) adult males, and (f) adult females of Paracartia grani feeding on Rhodomonas salina. Holling Type III curves were fitted to the clearance and ingestion rates as a function of the average prey concentration. Data in brackets are excluded from the model

Fig. 2 shows the power fits between copepod body mass and copepod F_{max} ($r^2 = 0.991$, $p < 0.01$; Fig. 2a) and I_{max} ($r^2 = 0.967$, $p < 0.001$; Fig. 2b). F_{max} of nauplii and copepodites increased with body mass with a scaling factor of 1.09 ± 0.07, whereas the fit between body mass and $I_{\max}$ had an exponent of 0.83 ± 0.05 considering all copepod life stages. $F_{\max}$ of adults did not follow the general pattern observed for nauplii and copepodites.

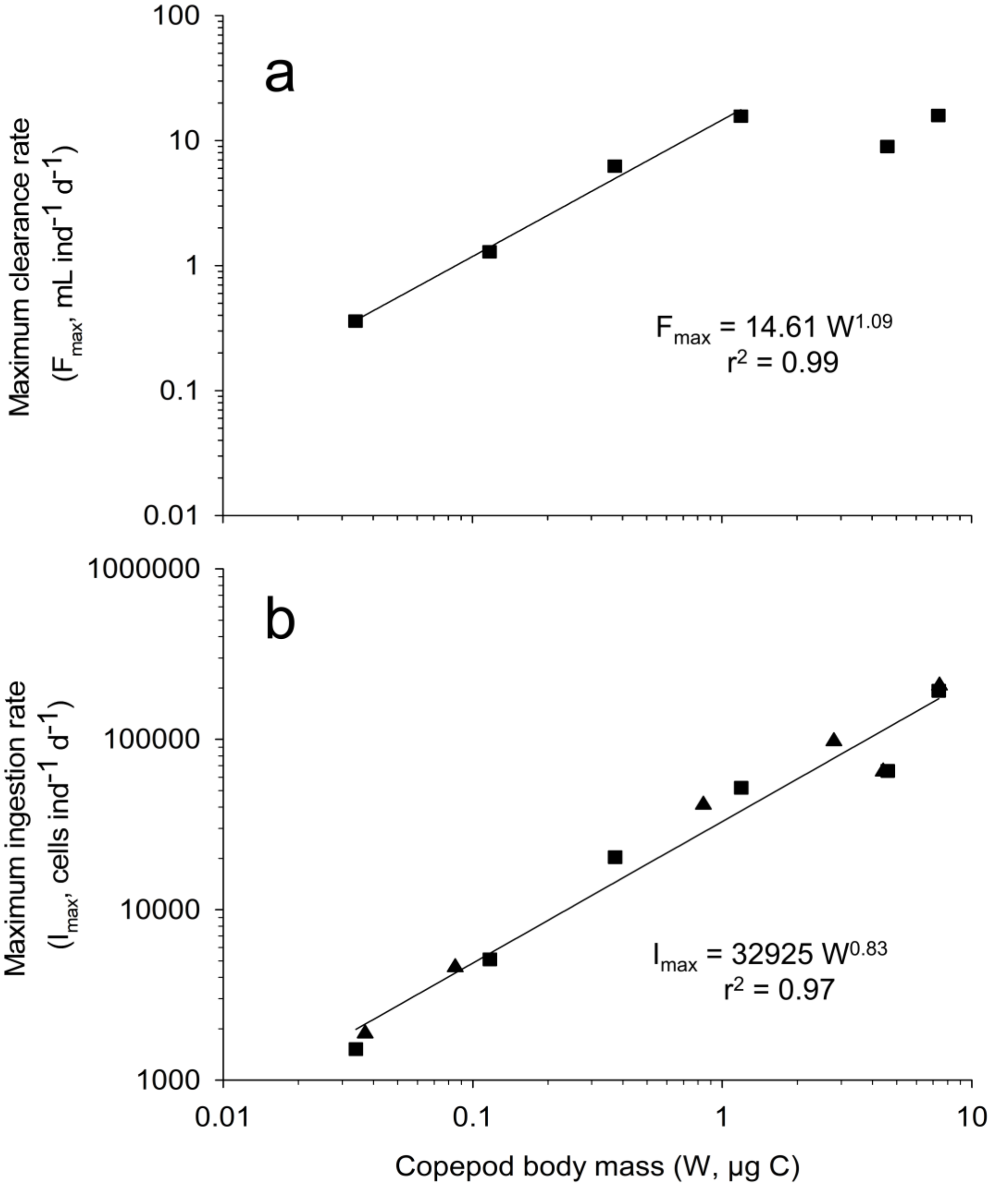

Fig. 2. (a) Maximum clearance rates (F_{max}) and (b) maximum ingestion rates (I_{max}) of Paracartia grani feeding on Rhodomonas salina with increasing copepod body mass (W). Power function fits and corresponding equations are shown. Note that for the clearance rates, only data on nauplii and copepodites are used for the fit. For the ingestion rates, data from the functional response experiments (■) and additional unpublished trials (▲) are pooled together

In terms of mass-specific rates, the maximum daily ingestion ranged from ca. 210% of the body carbon in nauplii to 265% of the body carbon in early copepodites and then declined to 68 and 124% of the body carbon in adult males and females, respectively (Fig. 3, Table 1). Adults became satiated at much higher prey concentrations than copepodites and nauplii: K_m values were 3773 cells ml^{-1} (ca. 170 µg C l^{-1}) and 6877 cells ml^{-1} (ca. 290 µg C l^{-1}) for males and females, respectively, and less than 2300 cells ml^{-1} (ca. 110 µg C l^{-1}) for juvenile stages (Table 1). The K_m values for copepodites (CI–II and CIII–IV) were lower than those for nauplii. The food concentrations at which 90% of satiation (i.e. the maximum ingestion rate) was attained (C_{90}) followed a similar pattern to that of K_m: decreasing from nauplii to copepodites and substantially increasing in adults (Table 1).

e) Discussion

Ontogenetic changes in the feeding functional response

We examined changes in the feeding functional response of the marine copepod *Paracartia grani* through ontogeny and focused on relevant ecological aspects, such as the shape and key parameters of the functional response (i.e. $I_{\max}$, $F_{\max}$, K_m). Previous studies have also addressed ontogenetic changes in feeding patterns of marine copepods, but they focused on other aspects of feeding, such as the prey size spectrum (Berggreen et al. 1988), or did not analyse functional responses through the whole developmental range from nauplii to adults (Almeda et al. 2010, Saiz et al. 2014).

The functional response of an organism shows its capability to exploit environmental resources and the potential flux of energy into secondary production (somatic growth, egg production). We found that the feeding functional response of *P. grani* grazing on *Rhodomonas salina* consistently followed a Holling Type III pattern in all life stages. Holling Type III responses have been traditionally linked to predator–prey systems where predators may control prey densities by switching to alternative prey types according to relative prey abundances, exploiting the more abundant resources and thereby ensuring a basal level of prey diversity in the system (Gismervik & Andersen 1997, Van Leeuwen et al. 2007).

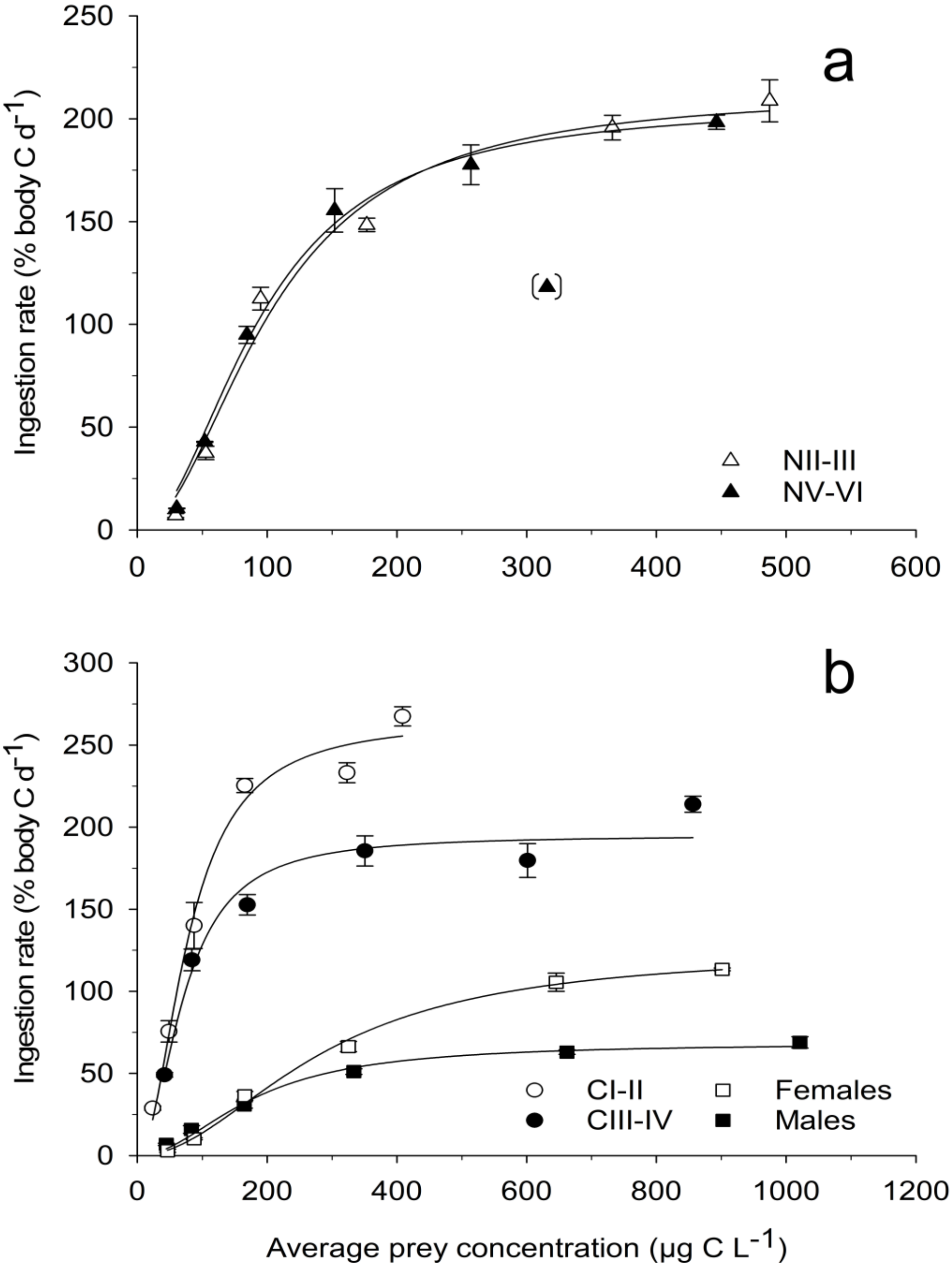

Fig. 3. Carbon-specific functional responses of (a) nauplii and (b) copepodites and adults of Paracartia grani feeding on Rhodomonas salina. Holling Type III curves were fitted to the carbon-specific ingestion rates as a function of the average prey concentration. Means and SE bars are shown. Data in brackets are excluded from the model

However, Holling Type III responses are not restricted to multiple-prey systems, as they have been reported in numerous single-prey experiments (Kiørboe et al. 2018). In our study, we conducted experiments with one algal species and inferred a clear Type III response in all cases. The interpretation of Type III responses in single-prey scenarios is that predators stop actively searching for prey when the energy gained by feeding would not compensate for the energy invested in capturing prey (in our study, this threshold would correspond to approximately 2000 cells ml^{-1} for nauplii and copepodites, and 4000–6000 cells ml^{-1} for adults). Accordingly, this foraging strategy has been proposed to be adaptive for copepods associated with productive environments where prey availability is high, such as coastal environments (Paffenhöfer & Stearns 1988). A recent analysis by Kiørboe et al. (2018) noted that in single-prey environments, Holling Type III responses are more typical of copepods with feeding strategies that involve higher metabolic costs (for instance, creating feeding currents to enhance the encounter rate with prey), while less active swimmers, such as ambush-feeding copepods, generally show a Holling Type II response. Adults of *Acartia* species (sensu lato) normally produce feeding currents when feeding on relatively small prey (Jonsson & Tiselius 1990, Saiz & Kiørboe 1995). The fact that we observed the same feeding functional pattern throughout the whole ontogeny of *P. grani* suggests that active foraging occurs not only in adults but also in the juvenile stages of this marine species. Nauplii of *P. grani* are known to perform continuous hopping movements during foraging and show a Type III response for a wide range of prey types (Henriksen et al. 2007, Helenius & Saiz 2017). There are no detailed reports regarding the underlying mechanisms involved in *P. grani* nauplii feeding, but in the case of the related species *Acartia tonsa*, nauplii are able to detect a prey item remotely and then capture it by using temporary feeding currents generated by the second antennae and mandibles (Paffenhöfer et al. 1996, Bruno et al. 2012).

Although previous studies (Almeda et al. 2010, Saiz et al. 2014) and our results indicate that the feeding functional response follows the same pattern through copepod development, Santer & van den Bosch (1994) reported ontogenetic differences in the freshwater copepod species *Cyclops vicinus*. They found that the naupliar and early copepodite stages of *C. vicinus* exhibited a Holling Type II response, whereas late copepodites and adults responded in more of a rectilinear pattern. However, their observations must be taken with caution because the rectilinear response in late copepodites and adults might be a consequence of not reaching satiating conditions in the experimental food concentrations. Moreover, it is often difficult to accurately distinguish between Type II and Type III responses in data sets, either because very low food concentrations might not be included in the experimental range or because the large variance in counts at low concentrations masks a Type III response.

Changes in F_{max} through ontogeny

The $F_{\max}$ that we estimated for nauplii II-III (0.36 ml ind.$^{-1}$ d^{-1}) was very similar to the value reported by Helenius & Saiz (2017) for the same copepod species, stage, and prey species (0.33 ml ind.$^{-1}$ d^{-1}). These values were also comparable to the $F_{\max}$ estimated by Henriksen et al. (2007) for *P. grani* nauplii II-III grazing on larger prey organisms (0.39 and 0.41 ml ind.$^{-1}$ d^{-1} for *Thalassiosira weissflogii* and *Heterocapsa* sp., respectively).

The $F_{\max}$ of *P. grani* increased with body mass from nauplii to late copepodites to the power of 1.09, which is in accordance with the scaling factor of 0.93 determined for the clearance rates of the closely related species *A. tonsa* when grazing on *Rhodomonas baltica* (Berggreen et al. 1988) and the value of 1 reported for $F_{\max}$ across various zooplankton taxa (Kiørboe 2011, Kiørboe & Hirst 2014). $F_{\max}$ describes the capability of a predator to locate and capture prey at low prey densities when factors such as handling time or gut filling are not relevant. Compared to nauplii, copepodites have more developed feeding appendages that can produce larger and stronger feeding currents to capture prey and scan larger volumes of water (Paffenhöfer 1971). Therefore, higher feeding efficiency in copepodites may result in a steeper increase in $F_{\max}$ with body mass compared to the typical allometric scaling factor of 0.75 found for other parameters, such as $I_{\max}$ (see Section 4.3).

The $F_{\max}$ of adults did not follow the general pattern observed in juvenile stages, as the $F_{\max}$ of males and females (ca. 9 and 16 ml ind.$^{-1}$ d^{-1}, respectively) were not higher than that of late copepodites (ca. 16 ml ind.$^{-1}$ d^{-1}). We think that this discordance in adult clearance rates is due to the relatively small size of the prey, *R. salina*. It is known that the optimal prey size of marine copepods changes with body size and through ontogeny (Berggreen et al. 1988, Landry & Fagerness 1988, Saiz et al. 2014). It could be argued that the use of the same prey type throughout development may affect the interpretation of our results. In fact, our estimates of the $F_{\max}$ for adult females were 4–7 times lower than those previously reported for *P. grani* females feeding on larger prey organisms, such as *T. weissflogii* (ca. 13 µm ESD, 70 ml female^{-1} d^{-1}; Saiz et al. 1992), *Heterocapsa* sp. (ca. 13 µm ESD, 66 ml female^{-1} d^{-1}; Isari et al. (2013), and *Oxyrrhis marina* (ca. 17 µm ESD, 111 ml ind.$^{-1}$ d^{-1}; Calbet et al. (2007). In Table 1, we can also see that adults showed the highest K_m and C_{90} values through development, indicating that the discrepancy between optimal prey size and predator size was the greatest among all life stages. Therefore, it is likely that if we had used larger prey in the experiments with adults, $F_{\max}$ would have conformed to the power-of-1 scaling pattern throughout development.

Changes in I_{max} through ontogeny

Although the $I_{\max}$ that we estimated for nauplii II-III was comparable to the value reported by Helenius & Saiz (2017) for the same copepod species, stage, and prey species (1623 and 1234 cells ind.$^{-1}$ d^{-1}, respectively), our estimate of the carbon-specific $I_{\max}$ (ca. 213% body C d^{-1}) was much higher than the value found in their study (74% body C d^{-1}). This large difference in mass-specific rates relies mostly on the fact that the carbon biomass that we extrapolated for our nauplii (34 ng C ind.$^{-1}$) was substantially lower than the measured biomass in their study (62 ng C ind.$^{-1}$), even though the average naupliar sizes were very similar (139 and 143 µm, respectively). Our estimate, nonetheless, was closer to the maximum daily ingestions reported by Henriksen et al. (2007) for *P. grani* nauplii feeding on *T. weissflogii* and *Heterocapsa* sp. (185 and 299% body C d^{-1}, respectively). In this regard, Henriksen et al. (2007) determined that *P. grani* nauplii, with an average body length of 165 µm, contained 40 ng C animal^{-1}, which indicates that the carbon content values measured by Helenius & Saiz (2017) might have been anomalously high.

The maximum daily ration that we estimated for adult females (124% body C) fell within the range of 103–188% d^{-1} found in the literature for the closely related species *A. tonsa* at same experimental temperature and when provided with the same prey species (Kiørboe et al. 1985, Toudal & Riisgård 1987, Støttrup & Jensen 1990, Thor & Wendt 2010). This value was also close to the specific rates reported for *P. grani* feeding on other prey, such as *Heterocapsa* sp. (ca. 13 µm ESD, 147% d^{-1}; Isari et al. 2013) and *Oxyrrhis marina* (ca. 17 µm ESD, 149% d^{-1}; Calbet et al. 2007). It is worth noting that across prey sizes, maximum mass-specific ingestion rates show a much narrower range of variability in comparison with F_{max}, which can differ by a factor of up to 7 (see Section 4.2). In this regard, Frost (1972) previously reported that the F_{max} of *Calanus pacificus* varied between 91 and 223 ml $ind.^{-1}$ d^{-1} across a range of prey diameters between 11 and 87 µm, whereas I_{max} remained fairly constant between 24.2 and 27.1 µg C $ind.^{-1}$ d^{-1}. F_{max} is very dependent on the predators' perception of prey, which is particularly sensitive to prey size and motility (Saiz et al. 2014, Almeda et al. 2018). In contrast, I_{max} appears to be not greatly influenced by prey size (Frost 1972) and is more constrained by factors such as copepod gut volume, digestion time, and gut turnover rate (Hassett & Landry 1988, Henriksen et al. 2007). Handling times, as a parameter in Holling equations, are typically also considered for the satiation effect, but in copepods they are generally too short to constrain food intake (Tiselius et al. 2013, Kiørboe et al. 2016). The fact that specific I_{max} values are not very dependent on prey size helps to explain the exponent of 0.83 in our allometric analysis. This exponent, although slightly higher, was not significantly different from the expected value of 0.75 (*t*-test, $p > 0.05$) according to the 3/4 power law accepted for most organisms, including marine calanoid copepods (West & Brown 2005, Saiz & Calbet 2007, Kiørboe & Hirst 2014). Thus, it appears that the use of a single prey size in our experiments did not result in any substantial bias in the estimation of I_{max}.

In relation to sex differences, mass-specific I_{max} values for males were ca. 1.8 times lower than those of females (68 and 124% of body C d^{-1}, respectively). Sex-related differences in feeding rates have been previously reported for the same copepod species (Saiz et al. 1992) and other copepod species (Saage et al. 2009, van Someren Gréve et al. 2017b). On the one hand, some authors have attributed the relatively lower feeding rates of males to size differences between the sexes

(Bautista et al. 1988, van Someren Gréve et al. 2017b). However, our allometric analysis (Fig. 2) shows that $I_{\max}$ values for *P. grani* males were lower than expected for a copepod of their size. In fact, the feeding rates of late copepodites were also higher than those of males. On the other hand, previous studies state that in some copepod species, adult males are more active swimmers than females and spend a substantial fraction of their time searching for a mate to the detriment of feeding (Kiørboe 2007, Almeda et al. 2017, van Someren Gréve et al. 2017a). We do not know whether sex-related differences in swimming activity could explain the low feeding rates found in *P. grani* males, but it seems unlikely given that the males and females of the related species *A. tonsa* appear to display similar swimming patterns (Bagøien & Kiørboe 2005). We think that the differences in maximum feeding rates between the sexes may not rely on their swimming behaviour but rather on differences in the allocation of energy to the reproductive process. In this regard, *P. grani* females can exhibit high reproductive output under optimal food conditions that would require higher ingestion rates (Saiz et al. 2015, Saiz et al. 2017).

f) Conclusions

Our study provides empirical data about the ontogenetic changes that marine copepods show in the exploitation of food resources. The insights obtained can help improve modelling capabilities to estimate energy transfer through copepod populations with different stage compositions in pelagic food webs. We have shown that the functional response of the copepod *Paracartia grani* when feeding on *Rhodomonas salina* followed a common pattern (Holling Type III) from early nauplii to adults. $F_{\max}$ increased with body mass with a power scaling factor of 1, except for the adult stages, which deviated from that common allometric trend, presumably due to the relatively small prey size. $I_{\max}$ scaled to body mass with a factor of 0.8, close to the typical value of 0.75 found in many animals. In this case, prey size did not seem to substantially affect our estimates of $I_{\max}$ along ontogeny. We suggest that future studies addressing feeding activity of copepods should also consider the changes in prey size spectrum along ontogeny. In addition, we think that plankton dynamic models would benefit from assessments in multiple resource scenarios, in which selectivity patterns and switching responses can modulate the foraging behaviour of marine copepods.

g) *Acknowledgements*

We thank Kaiene Griffell for assisting with culture maintenance. This study was framed within the project FERMI (CGL2014-59227-R, MINECO/AEI/FEDER, UE) and is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). M.O. was supported financially by an FPU grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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4.2. Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms

Reference:
Olivares, M.; Calbet, A.; Saiz, E. (2020). Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms. Aquatic Ecology, 54, pp. 697-709. https://doi.org/10.1007/s10452-020-09768-8

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a) Abstract

Marine planktonic copepods frequently exhibit diel feeding cycles coupled with vertical migrations. However, copepod feeding rhythms can be influenced by factors others than different food availability between layers. In this study, we determined the changes in the diel feeding behaviour of two marine copepod species (*Centropages typicus* and *Paracartia grani*) through multigenerational laboratory rearing, ontogeny, and upon the exposure to predator chemical cues. The wild females of both *C. typicus* and *P. grani* showed marked feeding rhythms with higher ingestion rates at night. The diel rhythms of *C. typicus* were maintained in the first laboratory-reared generation (F1), suggesting maternal effects, but disappeared in the following generations. The *P. grani* females of a long-term culture (> 10 years) also showed no differences in their day–night feeding activity. Ontogenetic variations were detected in the F1 generation of *C. typicus*: feeding rhythms were absent in naupliar stages, but adults fed more intensely at night. In the case of the cultured *P. grani*, in general none of the stages showed feeding rhythms. Laboratory-reared *C. typicus* (8–11 generations) did not recover back the natural cyclic feeding when exposed to jellyfish and fish exudates, indicating that either predation risk does not significantly affect their diel feeding activity or predator-induced responses in marine copepods might not involve chemical signalling. Our study confirms that feeding-related functional traits of marine copepods can experience quick multigenerational changes in the

laboratory; consequently, cultured copepods might not be good models for studies involving their diel feeding behaviour.

b) Introduction

Zooplankton are the main link between primary producers and higher trophic levels in pelagic systems (Turner 2004). They play a major role in nutrient recycling within the photic layer (Alcaraz et al. 1994) and contribute significantly to the vertical carbon export to deeper waters, either through sinking faecal pellets and carcasses, or through the active process of diel vertical migration (Longhurst et al. 1990; Longhurst and Harrison 1989; Zhang and Dam 1997). In terms of abundance and biomass, copepods commonly represent a large bulk within migrant mesozooplankton (Longhurst 1985). Migrant copepods typically feed in food-enriched upper layers at night and return to deeper waters during the daytime (Atkinson et al. 1992; Dagg et al. 1989). Thus, diel vertical migrations are frequently coupled with other activity rhythms (e.g. diel feeding cycles). However, feeding rhythms may also appear in non-migrant copepods and are not strictly linked to upward migrations into food-rich layers (Head et al. 1985; Stearns 1986). Therefore, the ultimate causes for copepod feeding rhythms might differ from those of vertical migrations.

Diel activity patterns of zooplankton are driven by light intensity changes and other factors such as predation risk, food availability, UV radiation, or temperature (Ringelberg 1995; Stearns and Forward 1984; Williamson et al. 2011). The relative importance of these factors to shape zooplankton diel patterns, however, depends on the species and habitats. In particular, levels of exposure and vulnerability to predators seem to highly influence the diel activity patterns of marine copepods. In this regard, Bollens and Frost (1989b) linked the amplitude of vertical migration in *Calanus pacificus* to the abundance of predatory fish. Also, Bollens and Frost (1991) and Bollens and Stearns (1992) found that the diel migrations and feeding rhythms of *Acartia hudsonica* became more pronounced in the presence of fish. Hence, even though prey availability and endogenous rhythmicity have been also proposed as driving factors of copepod diel rhythms (Huntley and Brooks 1982; Stearns 1986), the weakening or absence of diel rhythmicity in the activity of cultured copepods have been ultimately attributed to the lack of potential predators in the environment (Calbet et al. 1999). In this respect, the tracking of multigenerational changes in the diel behaviour of laboratory-reared marine copepods can provide new insights about the exogenous and/or endogenous causes that shape their diel activity patterns.

Copepods have mechanical and chemical sensors in their antennae that can be used to remotely detect predators (Hartline et al. 1996). However, it is not clear whether predator chemicals are really responsible for the predator-induced changes in copepod diel rhythms or, instead, other cues are involved. Cieri and Stearns (1999) and Cohen and Forward (2005b) found that the exposure to predator exudates induced, respectively, a lower daytime feeding in *Acartia tonsa* and *A. hudsonica*, and modifications in the vertical migration patterns of *Calanopia americana*. Contrarily, Bollens and Frost (1989a) and Bollens et al. (1994) reported that migration-related responses of *A. hudsonica* upon predator exposure did not involve chemical signalling. Therefore, the effects of predator chemical cues on copepod diel rhythms still remain debatable. In some cases the absence of responses to predator exudates could be argued with a lack of phenotypic plasticity in copepod populations (Kiørboe et al. 2018). In this respect, copepods reared in the laboratory in the absence of predators for several generations would be a good model to test the long-term phenotypic plasticity of copepod populations to respond to predation threat.

The diel behaviour of copepods can show significant changes through development. In relation to vertical migrations, Osgood and Frost (1994) and Fortier et al. (2001) reported that certain species show diel large-scale movements to deeper layers only during late stages, whereas early stages remain near the surface or are evenly distributed through the water column. Also, Atkinson et al. (1992) and Huntley and Brooks (1982) found that five species of copepods increased the amplitude of their vertical movements from nauplii to adults. Hence, smaller stages with lower swimming capacity might depend more on their reduced size to avoid visual predation, whereas larger stages would need to compensate their increased predation susceptibility during the daytime through downward migrations to deeper, darker layers (Buskey 1994; Neill 1992). It is not known, however, whether changes through development in diel migration patterns of marine copepods are associated to variations in their diel feeding behaviour. Although large marine copepods usually show higher feeding activity at night, it may well be that early development stages, closer in size to protozoan microzooplankton, find more advantageous to follow reverse diel feeding rhythms to avoid predation by adult copepods (Arias et al. 2017; Jakobsen and Strom 2004). Therefore, it can be hypothesized that copepod nauplii might show diel feeding patterns that are different from those of copepodites and adults.

Because of the key ecological role of copepods in aquatic ecosystems, the study of their feeding patterns and factors that modulate their diel behaviour is essential knowledge to improve our capability to determine the energy flux into secondary production in marine and freshwater environments. For that purpose, in this study we analysed the effects of multigenerational laboratory rearing, ontogeny and exposure to predator exudates on the diel feeding behaviour of two marine calanoid copepod species (*Centropages typicus* and *Paracartia grani*). Firstly, we monitored variations in the day and night feeding rates of field-collected (wild) and laboratory-reared descendant copepods. Secondly, we compared the diel feeding patterns of different copepod life stages from the same generation. Finally, we tested whether the presence of predator exudates (from both visual and non-visual predators) could trigger back diel feeding rhythms in copepod populations that had lost their natural rhythmic feeding after multiple-generation rearing.

c) *Material and methods*

Prey cultures

The algae *Rhodomonas salina* and *Heterocapsa* sp., and the heterotrophic dinoflagellate *Oxyrrhis marina* were used as prey for copepod cultures and experiments. The algae were grown in f/2 medium (Guillard 1983) and maintained in the exponential growth phase by diluting 1/3 of the cultures daily. *O. marina* was grown in Guillard's trace metal stock solution (Guillard 1983) and fed with *R. salina*. Cultures of *O. marina* were unfed for two days before the experiments to ensure that *R. salina* had been depleted when the copepod incubations started. All phytoplankton and microzooplankton were maintained in a climate-controlled room at 19 ± 1 °C and 10 h: 14 h light: dark photoperiod (average PAR during the daytime: 90 μmol m^{-2} s^{-1} for algae, 10 μmol m^{-2} s^{-1} for *O. marina*).

Copepod collection and cultures

Wild and laboratory-reared copepods of the species *Centropages typicus* and *Paracartia grani* were used for experiments. Wild specimens (F0 generation) were collected with a Juday-Bogorov plankton net fitted with 200-μm mesh in coastal waters off Barcelona (*C. typicus*, May 2017) and the harbour of El Masnou, a town located 20 km north of Barcelona (*P. grani*, October 2018). In the laboratory, adults of both species were sorted out with a pipette under a stereomicroscope and kept in 15-L polycarbonate tanks with 0.1-μm filtered seawater and food. Copepods were fed ad libitum with either *O. marina* (> 8 ppm, *C. typicus*) or *R. salina* (> 5 ppm, *P. grani*) for 1–2 days before experiments. The temperature and light regimes were those found at sea during sampling: 16 ± 1 °C and 13.5 h: 10.5 h light: dark cycle for *C. typicus*, and 19 ± 1 °C and 10 h: 14 h light: dark cycle for *P. grani*.

For laboratory-reared copepods, up to 11 new generations of *C. typicus* (F1–F11, approx. 1 month per generation) were produced from the eggs spawned by the collected wild specimens (F0) and the following generations (batches of 10,000–20,000 eggs per generation). Eggs were siphoned out from copepod tank bottoms and placed in new tanks with filtered seawater at same temperature and light conditions as the original cultures. These cohorts were fed ad libitum with *R. salina* and *O. marina* during naupliar stages, and with *O. marina* and *Heterocapsa* sp. during copepodite and adult stages. In the case of *P. grani*, the laboratory-reared cohort was obtained from a copepod stock culture kept at our institute for more than 10 years (Fn generation). This cohort was fed with *R. salina* through the entire development.

Experiments to assess the effect of multigenerational rearing on copepod feeding rhythms

The presence and magnitude of diel feeding rhythms (i.e. differences between day and night feeding rates) were analysed in wild (F0) and laboratory-reared (F1, F3, F5, F6, F8, F11) females of *C. typicus*, and wild (F0) and laboratory-reared (Fn) females of *P. grani*. In the case of *C. typicus*, two independent experiments were carried out for each studied generation, except for F5 (only one experiment).

Experiments consisted of paired day and night incubations with copepods and prey. Prior to incubations, copepods were collected from the stock cultures using a 200-µm sieve and transferred to filtered seawater for 1.5 h to ensure complete gut evacuation. Prey suspensions of *O. marina* (experiments with *C. typicus*) or *R. salina* (experiments with *P. grani*) were prepared at satiating or near satiation concentrations (Olivares et al. 2019; van Someren Gréve et al. 2017) using a Beckman Coulter Multisizer III particle counter. Two–three initial bottles (only prey), 3–4 control bottles (only prey) and 3–4 experimental bottles (prey and copepods) were filled with the prey suspensions; then, copepods were transferred into the experimental bottles using a wide-mouth pipette. The number of copepods per bottle was determined to ensure a ca. 20% decrease in the prey concentration by the end of the incubations. The prey and copepod concentrations in the experiments, as well as bottle volumes, can be found in Table 1. All the bottles were sealed with plastic wrap before capped to avoid air bubbles. Control and experimental bottles were mounted on a rotating plankton wheel (0.2 rpm; average PAR 21 µmol m^{-2} s^{-1} in the daytime incubations) and let incubate for 8–9 h in the respective day and night periods. Initial bottles were processed at the beginning of the incubations to determine the actual initial prey concentrations with the Coulter counter. At the end of the incubations, the contents of control and experimental bottles were sieved through a submerged 200-µm mesh to separate copepods from prey. Prey concentrations were then measured with the Coulter counter as for the initial bottles. Copepods were checked for survival and then fixed in 4% formaldehyde solution for counting and size determination. Average prey sizes were calculated as the geometric mean between initial and final volumes registered with the Coulter counter. Preserved copepods were photographed and prosome lengths were measured using the software package ImageJ (Schneider et al. 2012). The average prey and copepod sizes in the experiments are shown in Table 1.

Table 1 *Size and concentration of copepods (Centropages typicus, Paracartia grani) and prey (Oxyrrhis marina, Rhodomonas salina) in the feeding experiments*

Experiment	Copepod species	Generation	Copepod stage	Copepod size (µm)	Prey species	Prey ESD (µm)	Prey conc. (ppm)	Copepod conc. (cop bottle^{-1})	Bottle vol. (mL)
Multiple-generation rearing	*C. typicus*	F0	Females	977 ± 57	*O. marina*	16.8 ± 0.4	9.6 ± 0.4	33 ± 3	620
		F1–F11	Females	1032 ± 33	*O. marina*	17.1 ± 0.5	9.9 ± 0.4	32 ± 3	620
	P. grani	F0	Females	845 ± 2	*R. salina*	7.2 ± 0.02	4.7 ± 0.6	15 ± 1	320
		Fn	Females	1054 ± 2	*R. salina*	7.3 ± 0.03	4.6 ± 0.5	12 ± 3	320
Ontogeny	*C. typicus*	F1	NII–III	166 ± 14	*R. salina*	7.4 ± 0.20	3.6 ± 0.4	281 ± 46	72
		F1	NIV–V	224 ± 18	*R. salina*	7.4 ± 0.19	3.6 ± 0.4	171 ± 39	72
		F1	Males	964 ± 5	*O. marina*	16.7 ± 0.5	9.8 ± 0.4	38 ± 2	620
		F1	Females	1008 ± 7	*O. marina*	16.7 ± 0.5	9.7 ± 0.3	29 ± 2	620
	P. grani	Fn	NII–III	144 ± 2	*R. salina*	7.4 ± 0.10	2.3 ± 0.3	244 ± 44	72
		Fn	NIV–V	207 ± 0	*R. salina*	7.4 ± 0.09	2.3 ± 0.3	97 ± 28	72
		Fn	CII–III	573 ± 31	*R. salina*	7.0 ± 0.02	2.5 ± 0.3	56 ± 9	320
		Fn	CIV–V	803 ± 9	*R. salina*	7.3 ± 0.07	2.9 ± 0.2	57 ± 19	320
		Fn	Males	911 ± 10	*R. salina*	7.3 ± 0.04	4.6 ± 0.5	29 ± 5	320
		Fn	Females	1054 ± 2	*R. salina*	7.3 ± 0.03	4.6 ± 0.5	12 ± 3	320

The volume of the bottles is also provided

ESD equivalent spherical diameter, N nauplii, C copepodites. Means ± SD are shown

Experiments to assess ontogenetic changes in copepod feeding rhythms

Differences between day and night feeding rates were determined for nauplii II–III, nauplii IV–V and adults (males and females) of the F1 generation of *C. typicus*, and for nauplii II–III, nauplii IV–V, copepodites II– III, copepodites IV–V, and adults (males and females) of the long-term cultured *P. grani* (Fn generation). The experiments with *C. typicus* were carried out twice for each studied stage.

Experiments were conducted following the methodology described in the previous section. Details on prey and predator concentrations and bottle volumes are provided in Table 1. The way of adding copepods into experimental bottles differed depending on stage. Nauplii and copepodites were added as aliquots, and an equivalent volume of filtered seawater was added into the initial and control bottles to correct for any dilution effects. Adults were transferred to prey suspensions and added individually into the experimental bottles using a pipette. In the experiments with nauplii and copepodites, extra copepods were preserved in 4% formaldehyde solution at the beginning of the incubations to determine initial copepod size. At the end of the incubations, the contents of control and experimental bottles were filtered through submerged sieves (37 µm for nauplii, 60 µm for copepodites and 200 µm for adults) to separate copepods from prey. Copepods were preserved, posteriorly photographed, and then total (nauplii) or prosome (copepodites, adults) lengths were measured using ImageJ (Schneider et al. 2012). In the case of nauplii and copepodites, the arithmetic mean between initial and final sizes was computed, whereas only final length was considered for adults. The average sizes of prey and copepod stages in the experiments can be found in Table 1.

Experiments to assess the effect of the exposure to predator exudates on the feeding rhythms of cultured copepods

We carried out additional incubations in the experiments with the F8 and F11 females of *C. typicus* (described previously) to assess the effects of predator exudate exposure. These incubations included a control treatment with filtered seawater, and a treatment with a mixture of filtered seawater (75%) and water with predator exudates (25%). *O. marina* at 10 ppm was used as food source in all the incubations. In total, two experiments with jellyfish exudates (F8 females) and one experiment with fish exudates (F11 females) were conducted.

Water with jellyfish exudates was obtained from a 400-L aquarium containing 24 individuals of the jellyfish *Aurelia aurita* (umbrella diameter 4–6 cm) fed ad libitum with brine shrimps. Water with fish exudates was collected from a 2500-L tank (turnover rate of 2500 L h^{-1}) with 40 individuals of European bass (*Dicentrarchus labrax*, approx. 1200 g per individual) fed three times a week with fodder. The water with predator (jellyfish or fish) exudates was pre-filtered through a 20-µm sieve and then vacuum-filtered through a 0.2-µm nucleopore filter. Experimental copepods were acclimatized to one of the water types (i.e. with or without predator exudates) during four days before the experiments. During the acclimation period, 80% of the water was renewed daily in the copepod tanks to ensure that copepods were exposed to fresh predator chemical cues, as the exudates might degrade within 24 h (Dodson 1988; Loose et al. 1993).

Data analysis

Day and night copepod ingestion rates were calculated using the equations from Frost (1972). For carbon-specific feeding rates, the conversion factors 0.179 pg C μm^{-3} and 0.221 pg C μm^{-3} were applied for *O. marina* and *R. salina*, respectively (Helenius and Saiz 2017; Klein Breteler and Gonzalez 1986). To obtain copepod carbon weights, the dry weights of *C. typicus* were estimated using the length–weight relationship established for *Centropages hamatus* by Klein Breteler et al. (1982), and carbon weights were calculated as 40% of dry weights (Champalbert et al. 1973). In the case of *P. grani*, copepod carbon weights were obtained directly from the length–weight relationship reported for this species in Olivares et al. (2019). Total daily ingestions of *C. typicus* females were computed through weighing their day and night ingestion rates by the number of hours in each photoperiod.

A significance level of 0.05 was considered for all the statistical tests. Student's *t* tests were applied to check for significant differences between day and night carbon-specific ingestion rates within generations and life stages. Normality and homoscedasticity assumptions were checked with Shapiro–Wilk and Brown–Forsythe tests, respectively. When datasets did not pass normality or homoscedasticity tests, Mann–Whitney rank sum tests or Welch's *t* tests were applied, respectively. Because of the low number of replicates in the experiments with *C. typicus*, the data from independent experiments of each generation and copepod stage were pooled together for statistical tests. Linear regression was applied to check for any significant change in total daily ingestions of *C. typicus* females across generations. In the experiments with predator exudates, two-way ANOVA tests were run to assess the effect of the factors day/night and presence/absence of exudates on the ingestion rates of F8 and F11 females.

d) Results

Changes in copepod feeding rhythms through multigenerational laboratory rearing

The day and night ingestion rates of *C. typicus* females across multiple generations (F0–F11) are shown in Fig. 1a. F0 and F1 females showed, respectively, 63% and 51% significantly higher ingestion rates at night (two-tailed Student's *t* test, $p < 0.01$ for F0; two-tailed Mann–Whitney Rank Sum test, $p < 0.05$ for F1; Fig. 1a). Females of later generations (F3–F11), however, did not show any significant day–night difference in their feeding rates (two-tailed Student's *t* tests, $p > 0.05$ for F3, F6, F8 and F11; two-tailed Mann–Whitney Rank Sum test, $p > 0.05$ for F5; Fig. 1a). Thus, night/day ingestion rate ratios decreased from ca. 1.5 in F0 and F1 to ca. 1.0 in the next generations (Fig. 1b). Total daily ingestions ranged between 115 and 162% body C d^{-1} (average 134% body C d^{-1}) and did not significantly change across generations (linear regression, $p > 0.05$; Fig. 2).

Regarding the experiments with *P. grani* (F0 and F_n generations), the mean ingestion rates of wild females at night doubled those during the daytime (93.4 and 46.7 ng C_{prey} μg C_{cop}^{-1} h^{-1} for night and day, respectively; two-tailed Student's *t* test, $p < 0.001$; Fig. 3). The ingestion rates of cultured females were 21% higher at night but this day–night difference was not statistically significant (55.5 and

45.8 ng C_{prey} µg C_{cop}^{-1} h^{-1} for night and day, respectively; two-tailed Student's *t* test, *p* > 0.05; Fig. 3).

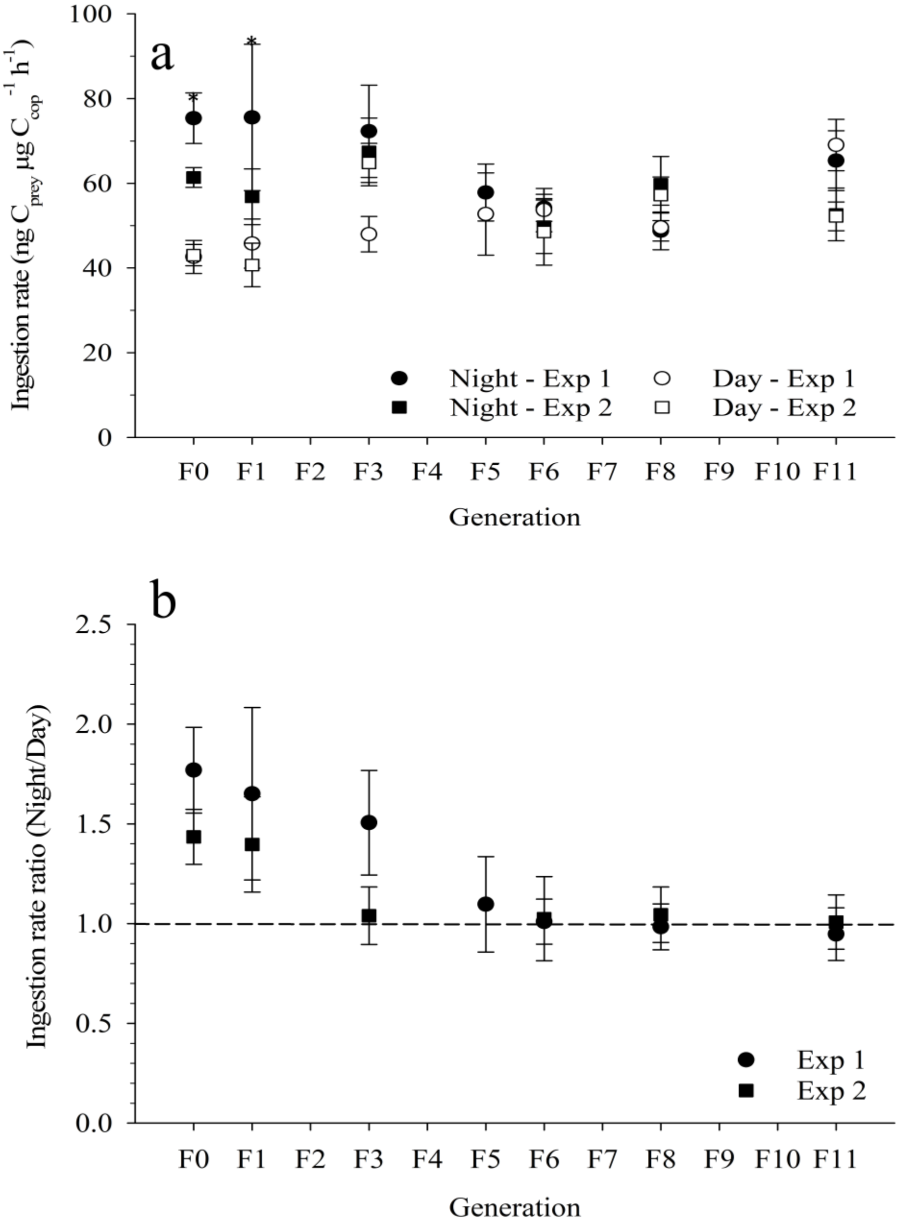

***Fig. 1 a** Day and night ingestion rates, and **b** night/day ingestion rate ratios of wild (F0) and laboratory-reared (F1–F11) females of Centropages typicus. Means and SE are shown. Significant differences between day and night ingestion rates are denoted with an asterisk (two-tailed Student's t test, p < 0.01 for F0; two-tailed Mann–Whitney Rank Sum test, p < 0.05 for F1). Dashed line indicates no difference between average night and day ingestion rates (ratio of 1.0)*

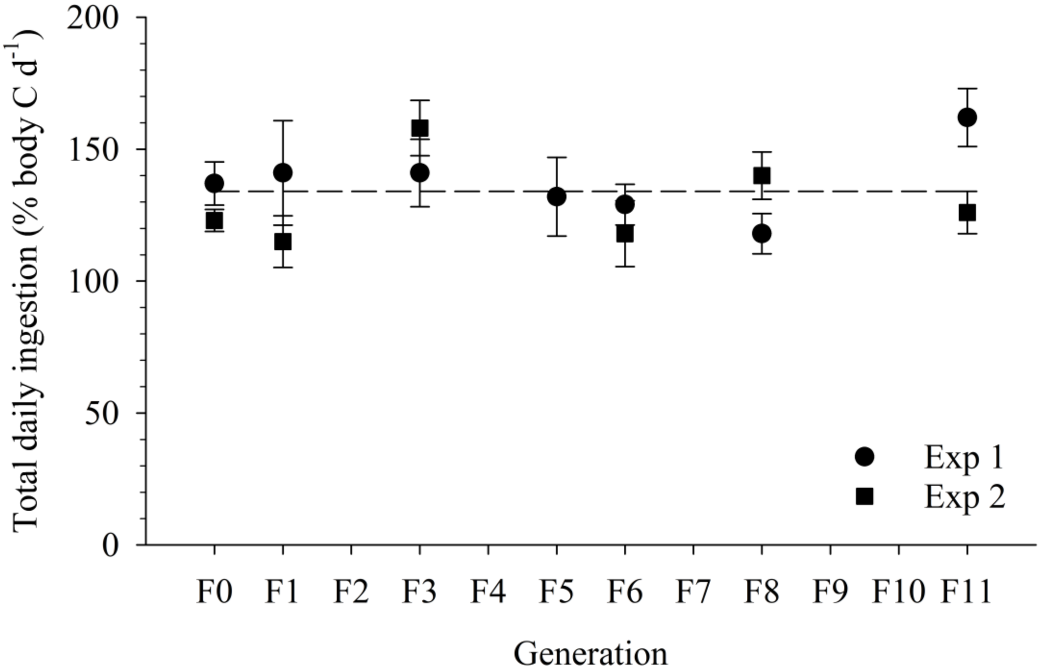

Fig. 2 *Total daily ingestions of wild (F0) and laboratory-reared (F1–F11) females of Centropages typicus. Means and SE are shown. Dashed line represents the average daily ingestion across generations (134% body C d^{-1})*

Ontogenetic changes in copepod feeding rhythms

The diurnal and nocturnal feeding rates of nauplii and adults of *C. typicus* (F1 generation) are shown in Fig. 4a. The mean ingestion rates of NII–III and NIV–V did not show any significant difference between day and night (two-tailed Student's *t* tests, $p > 0.05$; Fig. 4a). However, the mean ingestion rates of adults were about 50% significantly higher at night for both sexes (two-tailed Mann-Whitney rank sum test, $p < 0.05$ for females; one-tailed Welch's test, $p < 0.05$ for males; Fig. 4a).

In the experiments with the long-term cultured *P. grani* (Fn generation), in general, none of the stages showed any significant difference between their day and night feeding rates (two-tailed Student's *t* tests, $p > 0.05$ for CII–III, CIV–V, males and females; two-tailed Mann–Whitney Rank Sum test, $p > 0.05$ for NIV–V; Fig. 4b). The only exception was found in nauplii II–III, which exhibited 23%

significantly higher ingestion rates at night (day: 85 ± 1.7 ng C_{prey} µg C_{cop}^{-1} h^{-1}, night: 105 ± 1.2 ng C_{prey} µg C_{cop}^{-1} h^{-1}; Student's *t* test, $p < 0.001$; Fig. 4b).

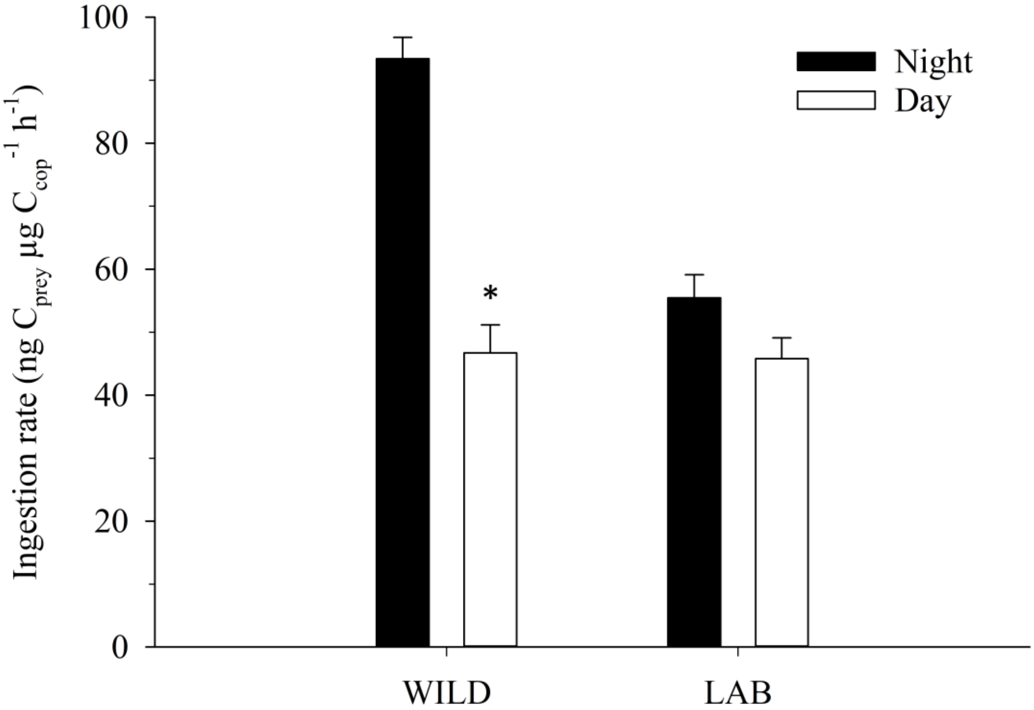

***Fig. 3** Day and night ingestion rates of wild ("WILD") and laboratory-reared ("LAB") females of Paracartia grani. Means and SE are shown. Significant differences between day and night ingestion rates are marked with an asterisk (two-tailed Student's t test, p < 0.001). Notice that average copepod sizes were 845 µm and 1054 µm for wild and cultured specimens, respectively*

Effect of exposure to predator exudates on the diel feeding rhythms of cultured copepods

The mean ingestion rates of F8 and F11 females of *C. typicus* exposed to predator exudates ranged between 49.8 and 69.1 ng C_{prey} µg C_{cop}^{-1} h^{-1} and did not show any significant difference to those of the non-exposed females (two-way ANOVA, *p* > 0.05; Fig. 5).

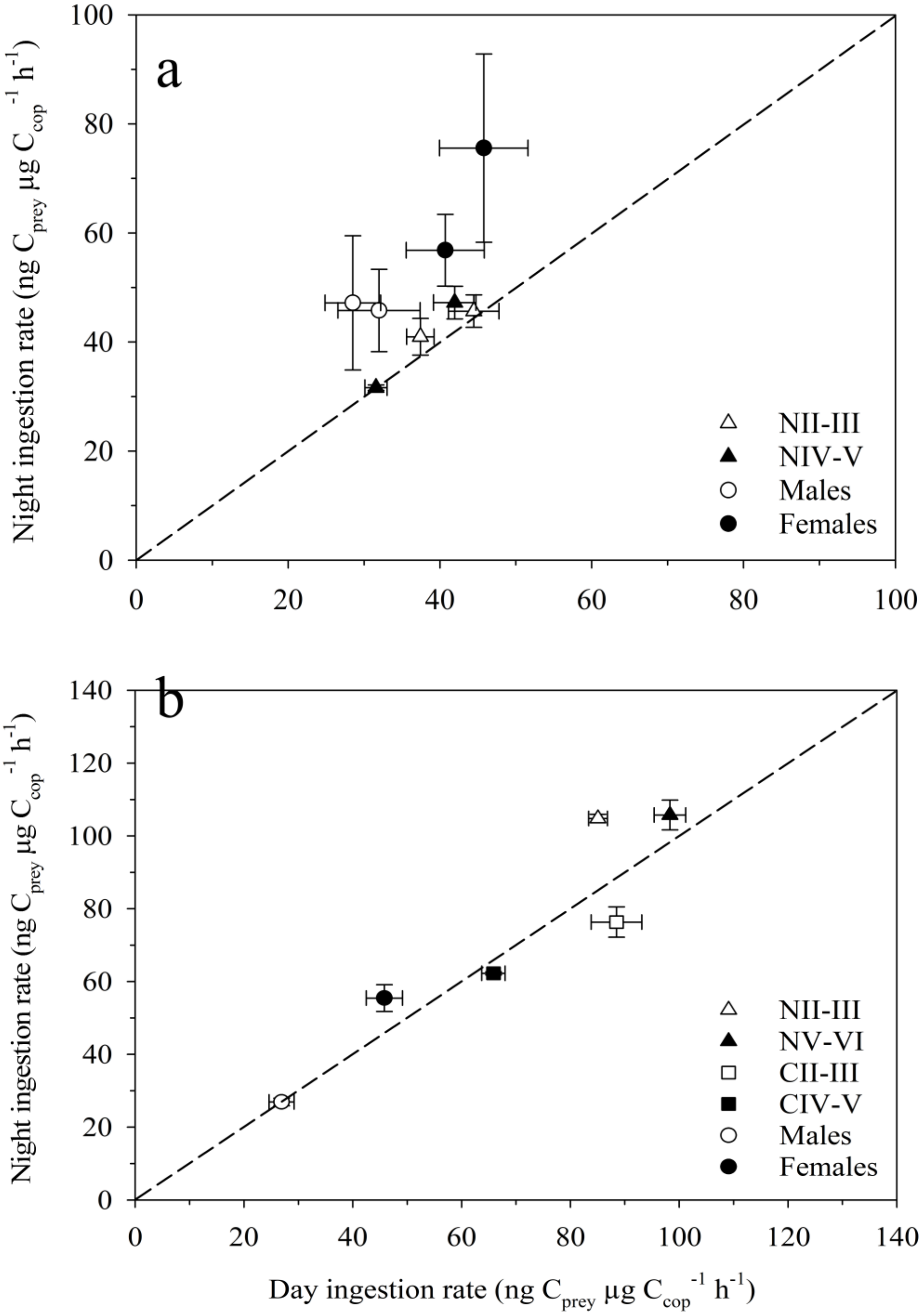

Fig. 4 *Day and night ingestion rates of* ***a*** *nauplii (N) and adults of Centropages typicus and* ***b*** *nauplii (N), copepodites (C), and adults of Paracartia grani. Means and SE are shown. Dashed line indicates no difference between average day and night ingestion rates*

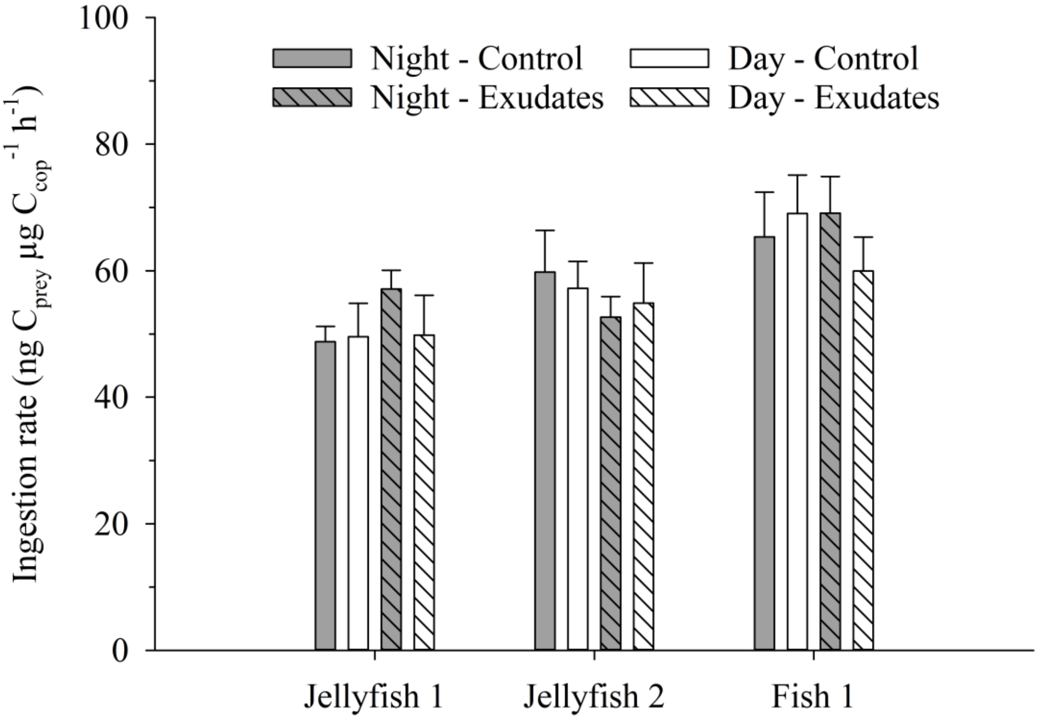

Fig. 5 *Day and night ingestion rates of laboratory-reared females of Centropages typicus incubated without predator exudates ("control") and with predator exudates ("exudates"). Two experiments with jellyfish exudates and one experiment with fish exudates are shown. Means and SE are provided*

e) Discussion

Loss of copepod feeding rhythms through multigenerational rearing

In our experiments the natural feeding rhythms of the marine copepods *C. typicus* and *P. grani* were lost through multigenerational laboratory rearing. In the case of *C. typicus*, the absence of rhythms was manifest after only 2–3 generations. Strong positive selection can lead to significant genotypic (and phenotypic) shifts in copepod populations, especially upon exposure to (or lack of) environmental stressors (Jiang et al. 2011; Sun et al. 2014). In our study, the quick loss of diel rhythms could reflect a sharp selection of phenotypes that show constant and continuous feeding when food availability is high. As evidenced in experiments with intermittent food supply (analogous to feeding cycles, i.e. fluctuations in food intake), rhythmic feeding might imply certain fitness costs (Calbet and Alcaraz 1996; Nival et al. 1990). Still, there are copepods that can counteract the negative effects of non-continuous feeding through enhancing food intake after starvation periods (Kremer and Kremer 1988; Runge 1980). In this regard, Davis and Alatalo (1992) reported that *C. typicus* is indeed capable to cope with short-term food fluctuations (0.5–1 days) in terms of survival, growth, development, and egg production rates. In agreement with this hypothesis, we found that total daily ingestions of *C. typicus* females remained invariable across generations regardless diel feeding behaviour. Thus, it seems unlikely that a strong phenotype selection could have caused the phenotypic shift in our study, as the potential adaptive value of non-rhythmic feeding (i.e. higher daily consumption) was not inferred.

Among the factors that can affect diel activity rhythms in copepods, predation threat has acquired the highest acknowledgement, especially regarding vertical migrations (Bollens and Frost 1989b; Frost and Bollens 1992). In relation to feeding patterns, Bollens and Stearns (1992) found that the amplitude of the diel feeding rhythms of *Acartia hudsonica* greatly increased in the presence of zooplanktivorous fish. Thus, the loss of feeding rhythms through multigenerational rearing could be attributed to the absence of predators in the environment. However, if copepod feeding rhythms were exclusively dependent on predator exposure, we would not expect to find any day–night difference in the food intake of copepods reared in complete predator-free conditions. Yet, we found that the first generation of laboratory-reared females (F1) still exhibited a marked diel feeding cycle despite of being never exposed to predators. Therefore, other factors beyond direct exposure to predators seem to be involved in the modulation of copepod diel rhythms.

Light conditions, prey availability or endogenous rhythmicity could also influence the diel feeding behaviour of marine copepods. About the former, we tried to simulate the light conditions that copepods experience in nature (not only photoperiod, but also light intensity). Thus, the average light intensity in the daytime incubations (ca. 20 µmol m^{-2} s^{-1}) was in the range of the typical values found at the depth where copepods remain during the daytime in the NW Mediterranean (Alcaraz 1988; Estrada 1985). Therefore, we do not think that the experimental light conditions could have caused any significant shift in the diel feeding rhythms of copepods. Regarding food conditions, copepods may show feeding rhythms even at low prey concentrations (Durbin et al. 1990). Still, higher prey availability normally translates into larger amplitudes of both vertical migrations and feeding cycles (Calbet et al. 1999; Huntley and Brooks 1982). In this regard, we kept copepods well-fed through development and experiments, so the magnitude of feeding rhythms should have been kept maximized. We do not know, however, whether the lack of rhythmicity in food availability (as it usually occurs in nature) could have contributed anyhow to the loss of natural rhythmic feeding in copepods. Finally, it is also worth noticing that circadian clocks have been reported to drive the expression of activity rhythms in marine copepods, particularly in vertical migrations (Cohen and Forward 2005a; Häfker et al. 2017) and feeding cycles (Olsen et al. 2000; Stearns 1986). However, the quick disappearance of diel feeding rhythms in our experiments suggests that exogenous cues may play a more important role in this phenotypic response.

The maintenance of diel feeding rhythms in the first reared generation (F1) of *C. typicus* suggests the non-genetic transmission of certain phenotypes through epigenetic mechanisms (Uller 2008). Previous studies with zooplankton demonstrated that exposure to non-lethal chemical cues (e.g. predator or conspecific cues) can lead to transgenerational changes in the functional traits of non-exposed offspring, such as helmet formation or earlier maturation in *Daphnia* (Agrawal et al. 1999; Walsh et al. 2015). Epigenetic mechanisms that mediate non-genetic inheritance have been already investigated in daphnids (Robichaud et al. 2012; Schield et al. 2016), but remain largely unexplored in other zooplankton groups. A recent study by Guyon et al. (2018) reported epigenetic responses (DNA methylation) of copepods to toxic exposure. It seems plausible, therefore, that the presence of diel feeding rhythms in the F1 generation of *C. typicus* could reflect epigenetic modifications determined by the external context experienced by predecessors (F0) in natural conditions (maternal effects).

Lack of response to predator chemical cues

We found that the exposure to predator exudates did not induce the recovery of diel feeding rhythms in laboratory-reared copepods (F8–F11 generations). If predation threat was pivotal to trigger rhythmic feeding in marine copepods, multigenerational rearing could have led to lower intrapopulation plasticity in the response to predatory signals (Cousyn et al. 2001). The loss of capacity to react to predator chemical cues would bring ecologically relevant consequences for species interactions and, at the end, community dynamics (Miner et al. 2005). However, it could happen that predator-induced responses of marine copepods might not involve chemical signalling.

There is a large bulk of research about the significant effects of kairomones on freshwater zooplankton (Lass and Spaak 2003). However, with very few exceptions (e.g. Cieri and Stearns (1999), Cohen and Forward (2005b)), the attempts to use predator chemical cues to induce behavioural changes in marine copepods have failed (Bollens et al. 1994; Kiørboe et al. 2018). This suggests that, unlike in freshwater zooplankton, responses of marine zooplankton to predation threat might be mediated by non-chemical cues (Kiørboe et al. 2018; Ringelberg 1995), whereas chemoreception would be more decisive for other purposes such as food selection or mate finding (Bagøien and Kiørboe 2005; Buskey et al. 2011; Tiselius et al. 2013). We cannot discard, however, that copepod feeding rhythms might not be that influenced by predation threat, and/or that the predators selected for our experiments (jellyfish *Aurelia aurita* and fish *Dicentrarchus labrax*) were unsuitable for the goal of our study. Thus, a non-visual predator like *A. aurita* might not alter copepod diel cycles that might be essentially triggered by visually guided predators (Bollens and Stearns 1992). Regarding *D. labrax*, the diet of adults certainly shows a lower preference over copepods compared to that of juveniles (Rogdakis et al. 2010). In fact, non-lethal effects of predation in copepods and other zooplankters are highly predator-specific (Lüning 1992; Ohman 1990) and may affect behaviour in very different ways (Saiz et al. 1993).

Ontogenetic changes in copepod feeding rhythms

Diel feeding rhythms were absent in naupliar stages but appeared in adults of the F1 generation of *C. typicus*. These ontogenetic variations agreed with diel migration patterns, as nauplii either do not migrate or conduct weaker vertical migrations compared to late life stages (Atkinson et al. 1992; Fortier et al. 2001;

Huntley and Brooks 1982). The higher predation susceptibility of young stages to non-visual predators (Titelman 2001), more active at night, makes nocturnal feeding much less adaptive to nauplii than to copepodites and adults (Boersma et al. 2014; Ohman 1990; Uye and Liang 1998). Moreover, the lower tolerance of early stages to fluctuations in food intake (Calbet and Alcaraz 1997) indicates that the appearance of feeding rhythms in marine copepods might be dependent on stage-specific metabolic requirements.

Regarding the long-term cultured *P. grani* (> 10 years in the laboratory), in general none of the stages showed any difference in their day–night feeding activity. Contrarily, wild adults of this species typically show higher feeding rates at night (this study; Bautista et al. (1988); Calbet et al. (1999)). As demonstrated in our experiments, laboratory-reared copepods might not conserve their natural rhythmic feeding behaviour after multigenerational rearing. In the case of nauplii II–III, we found an unexpected night-skewed feeding, although the amplitude of such a rhythm was low (23% higher ingestion rates at night). We analysed closely the initial and final stage compositions in that experiment (data not shown in the paper) and found that the stage proportions were really similar at the beginning of the day and night incubations (ca. 90% NII and 10% NIII). However, the final stage composition differed slightly between incubations (50:50 and 40:60 NII:III proportions during the daytime and night incubations, respectively). One could speculate that NII ingestion rates could have been comparatively lower if any yolk remains were still available. Therefore, the day–night differences found in that experiment could be attributed to the larger proportion of NIII at the end of the night incubation.

f) Conclusions

Our study confirms that feeding-related functional traits of marine copepods can show rapid multigenerational changes in the laboratory. The quick loss of natural rhythmic feeding through rearing suggests that marine copepods might express feeding rhythms upon exogenous cues, and not for endogenous causes, which may bring concern for the use of cultured copepods in experiments involving their diel feeding behaviour. The maintenance of diel rhythms in the first laboratory-reared generation of *C. typicus* indicates epigenetic mechanisms that mediate the non-genetic transmission of phenotypic traits (maternal effects). The evidence of ontogenetic variations in diel feeding behaviour stands out the importance of age-related factors, such as size-dependent vulnerability to predation, for the appearance of diel rhythms in copepods. Finally, the lack of response to the exposure of predator exudates indicates that either predator-induced responses of marine copepods might not involve chemical signalling, or factors other than predation risk can act as driving factors of copepod feeding rhythms.

g) Acknowledgements

We would like to thank Kaiene Griffell   for assisting with field sampling and culture maintenance. We also want to thank Ainara Ballesteros and Mercedes Blázquez for allowing the collection of water with jellyfish and fish exudates. This study was framed within the Project FERMI (CGL2014-59227-R, MINECO/AEI/FEDER, UE), and is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). Manuel Olivares is supported financially by a FPU Grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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4.3. Non-lethal effects of the predator *Meganyctiphanes norvegica* and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod *Centropages typicus*

Reference:
Olivares, M.; Tiselius, P.; Calbet, A.; Saiz, E. (2020). Non-lethal effects of the predator *Meganyctiphanes norvegica* and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod *Centropages typicus*. Journal of Plankton Research, 42(6), pp. 742-751.
https://doi.org/10.1093/plankt/fbaa051

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a) Abstract

Predators can induce changes in the diel activity patterns of marine copepods. Besides vertical migration, diel feeding rhythms have been suggested as an antipredator phenotypic response. We conducted experiments to assess the non-lethal direct effects of the predator *Meganyctiphanes norvegica* (northern krill) on the diel feeding patterns of the calanoid copepod *Centropages typicus*. We also analysed the influence of seasonal photoperiod and prey availability on the intensity of copepod feeding rhythms. We did not detect any large effect of krill presence on the diel feeding behaviour of copepods, either in day-night differences or total daily ingestions. Seasonal photoperiod and prey availability, however, significantly affected the magnitude of copepod feeding cycles, with larger diel differences in shorter days and at lower prey concentrations. Therefore, the role of non-lethal direct effects of predators on the diel feeding activity of marine copepods remain debatable and might not be as relevant as in freshwater zooplankton.

b) *Introduction*

Predation threat can trigger a wide variety of responses in animals, such as changes in morphology, physiology and/or behaviour (Lima, 1998; Agrawal, 2001). These predator-induced phenotypic changes have evolved to increase the animal's survival chances, but also bring certain fitness costs in terms of energy acquisition and resource allocation (Sih, 1980; Lima and Dill, 1990; Preisser *et al.*, 2005). Thus, predators can have negative impacts on prey populations not only through direct predation (consumptive effects), but also through the so-called non-consumptive (or non-lethal) effects. Non-lethal effects of predation can even represent a higher cost for prey demography than predation itself (Preisser *et al.*, 2005), and certainly have important ecological implications regarding community dynamics (Werner and Peacor, 2003; Schmitz *et al.*, 2004).

Within marine communities, copepods are a vital link between primary producers and fish (Runge, 1988) and typically account for the highest abundance and biomass among mesozooplankton (Longhurst, 1985). Therefore, non-lethal effects of predators on copepod populations can translate into important cascading effects in marine food webs (van Someren Gréve *et al.*, 2019). Copepod responses to increasing predation risk include changes in their foraging time, swimming speed and reproductive output (Saiz *et al.*, 1993; van Duren and Videler, 1996; Lasley-Rasher and Yen, 2012; Heuschele *et al.*, 2014). Of particular relevance is how predation risk can alter copepod diel behaviour. For instance, predation threat appears to be the major driver of diel vertical migrations in marine copepods (Frost, 1988; Ohman, 1990; Bollens and Frost, 1991; Hylander and Hansson, 2013). Migrant copepods typically stay in food-enriched upper waters at night, and move to deeper, darker layers during the daytime to avoid visual predation.

Besides vertical migration, copepods frequently show other diel activity rhythms involving their feeding, spawning and moulting patterns (Ohman, 1988). About the former, copepods generally show higher feeding activity at night (Atkinson *et al.*, 1992; Dagg *et al.*, 1998). Nocturnal feeding is usually coupled to vertical migrations, but this feeding behaviour is not necessarily a consequence of staying in food-enriched upper layers at night. In fact, rhythmic feeding may also appear in non-migratory copepods (Hayward, 1980; Head *et al.*, 1985). Therefore, diel feeding rhythms of marine copepods might confer an adaptive advantage that is independent of vertical migration.

Feeding in copepods implies higher motility and conspicuousness, which increases their detectability and predation vulnerability (Tsuda *et al.*, 1998; Uttieri *et al.*, 2013; Kiørboe *et al.*, 2014). This especially applies to daylight hours when copepods are more susceptible to visual predation (Tsuda *et al.*, 1998; Torgersen, 2001). Thus, feeding rhythms (i.e. lower daytime activity) have been traditionally considered an antipredator strategy in copepods (Ohman, 1988). Bollens and Stearns (1992) and Cieri and Stearns (1999) found that the planktonic copepods *Acartia tonsa* and *Acartia hudsonica* showed a lower daytime gut fullness when exposed to fish or fish exudates. However, other studies have not found any effect of predation threat on the feeding behaviour of marine copepods (Kiørboe *et al.*, 2018; Olivares *et al.*, 2020). Hence, the predator effects on copepod feeding rhythms still remain unclear. Most former research on this topic relied on predator exudates as predatory signals, even though marine copepods are known to respond to hydromechanical cues generated by predators (Kiørboe *et al.*, 1999; Hwang and Strickler, 2001; Buskey *et al.*, 2011). In this respect, further experiments with copepods exposed to freely swimming predators are necessary to detect predator-induced responses that are not only chemically triggered (e.g. Saiz *et al.* 1993; Tiselius *et al*. 1997).

The intensity of feeding-related antipredator responses of copepods (e.g. feeding rhythms) can depend on other factors besides predation threat. Copepod diel rhythms can show great seasonal variations (Durbin *et al.*, 1995; Irigoien *et al.*, 1998). These seasonal differences could be attributed to changes in the relative length of daylight periods associated to a higher predation threat (Lima and Bednekoff, 1999). Also, copepods can adapt their foraging behaviour to changing food conditions that affect their risk of being predated (Tiselius *et al.*, 1997; Visser, 2007; van Someren Gréve *et al.*, 2019). However, the effect of increasing food availability on copepod feeding rhythms is controversial. For instance, Hassett and Blades-Eckelbarger (1995) found that day-night differences in copepod feeding activity became larger at lower prey concentrations, whereas Calbet *et al.* (1999) reported that lower food concentrations did not affect or led to weaker diel feeding rhythms in some species.

The main goal of our study was to determine the non-lethal effects of predators on the diel feeding behaviour of marine copepods. Additionally, we also analysed the influence of seasonal photoperiod and prey availability on the magnitude of copepod feeding rhythms. We conducted laboratory experiments with the calanoid copepod *Centropages typicus* and the krill *Meganyctiphanes norvegica* as predators. *M. norvegica* acts as a key predator and grazer in pelagic communities of the North Atlantic with a preference for large and medium-sized copepods (Beyer, 1992; Båmstedt and Karlson, 1998; Agersted and Nielsen, 2016), and is a fundamental prey item for larger fish, squids and whales (Schmidt, 2010; Simard and Harvey, 2010; Suca *et al.*, 2018).

c) Method

Experimental organisms

The heterotrophic dinoflagellate *Oxyrrhis marina*, the calanoid copepod *Centropages typicus* and the euphausiid *Meganyctiphanes norvegica* were used for experiments in summer 2018 and autumn 2019.

O. marina was grown in 0.5-µm filtered seawater at 18±0.5°C and fed daily with the cryptophyte *Rhodomonas salina*. *R. salina* was grown in B medium (experiments in 2018, Hansen (1989)) or f/2 medium (experiments in 2019, Guillard (1983)). The cultures of *O. marina* were not fed for 48 h before experiments to ensure the absence of *R. salina* cells during incubations.

Copepods were collected in the Gullmar Fjord (58° 15.7' N, 11° 26.7' E, Sweden) using a 250-µm mesh plankton net. In the laboratory adult females of *C. typicus* were isolated using a pipette and kept in 8-L polycarbonate tanks with filtered seawater and food (*O. marina*, > 4 ppm). The sorted copepods were maintained at 14.5±0.5°C under a photoperiod that simulated natural light conditions: 16 h: 8 h light: dark in summer, and 10 h: 14 h light: dark in autumn.

Krill (*M. norvegica*) were collected in the deepest part of the fjord (58° 19.0' N, 11° 32.6' E) using an Isaacs-Kidd Midwater Trawl. Upon arrival at the station, the specimens were transferred to a 300-L glass fibre flow-through tank at 10°C and turnover rate 450 L h^{-1}. Krill were kept in constant darkness and fed daily with freshly collected zooplankton from the fjord.

Experimental set-up

Experiments consisted of day and night incubations of copepods (*C. typicus*) feeding on *O. marina* in the absence and the presence of predators (krill *M. norvegica*). Before incubations, copepods were collected from their maintenance tanks using a 200-µm mesh sieve and placed in filtered seawater for ca. 1.5 h to allow gut evacuation. The cell concentration of *O. marina* stock culture was determined with a Z Series Coulter Counter. About 8 to 10 bottles were filled with filtered seawater and *O. marina* was added to each bottle to obtain final prey concentrations of either 5.5–7 ppm (ca. 1900–2700 cells mL^{-1}; high food; five experiments) or 1.0 ppm (ca. 250 cells mL^{-1} ; low food; one experiment). The bottle volumes and *O. marina* concentrations in the experiments are shown in Table I. Four of the bottles were used as control bottles (*O. marina* + copepods) and four to six bottles as experimental bottles (*O. marina* + copepods + krill). A total of 30 copepods and one krill were added to each experimental bottle using, respectively, a wide-mouth pipette and an aquarium fish net (except for one experiment, with only 20 copepods per bottle). The copepod densities in the bottles (7.5–13 cop L^{-1}) were comparable to those that can be found in the Gullmar Fjord (Vargas *et al.*, 2002; Tönnesson and Tiselius, 2005). In the case of krill, the experimental densities (0.25–0.4 ind L^{-1}) were higher than the typical average densities of *M. norvegica* in nature (Onsrud and Kaartvedt, 1998; Tarling *et al.*, 1998), but fell within the range of densities reported for dense krill swarms (Nicol, 1986; Kaartvedt *et al.*, 2005). The bottles were then incubated for 8.5–11.5 h in a temperature-controlled room at 14.5±0.5°C and under the seasonal photoperiod specified before (Table I). The bottles were lit from the side to diminish vertical heterogeneity in the distribution of *O. marina* and *C. typicus* due to small-scale migrations during incubations (Alcaraz *et al.*, 2007; Bochdansky *et al.*, 2010; Bollens *et al.*, 2011). After the incubations, the contents of the bottles were sieved through a 200-µm mesh to collect copepods and krill, and then through a 20-µm mesh to collect copepod faecal pellets. The survival of copepods and krill was checked and the number of dead copepods was noted. The bottles with dead krill were discarded for data analysis (2 out of 30 bottles). The length of krill specimens was measured with a ruler. Copepods and faecal pellets were preserved with Lugol's solution for number and size determination. Photos of 20 copepods and 60–70 faecal pellets were taken per treatment (i.e. with and without krill), and the prosome length of copepods and the length and width of faecal pellets were measured with the software ImageJ (Schneider *et al.*, 2012). *O. marina* size was obtained from Coulter Counter data registered at the beginning of the incubations.

Table I: Temperature, light conditions, concentrations of prey (Oxyrrhis marina) and copepods (Centropages typicus) and bottle volumes used in the experiments. Mean ± SD are provided

Experiment	Date	Temperature (°C)	Photoperiod (day: night)	Irradiance (µmol photons m^{-2} s^{-1})	Prey conc. (ppm)	Copepods per bottle	Bottle volume (mL)
1	12 Aug	14.7 ± 0.23	16h: 8h	2.2 ± 0.21	5.5	20	2300
2	16 Aug	14.7 ± 0.23	16h: 8h	2.2 ± 0.21	7.0	30	2300
3	24 Aug	14.7 ± 0.23	16h: 8h	2.2 ± 0.21	6.0	30	2300
4	18 Oct	14.4 ± 0.11	10h:14h	1.7 ± 0.33	6.5	30	4000
5	25 Oct	14.4 ± 0.11	10h:14h	1.7 ± 0.33	1.0	30	4000
6	28 Oct	14.4 ± 0.11	10h:14h	1.7 ± 0.33	6.5	30	4000

The number of replicates was determined based on power calculations and published data on variability. For the predation by krill, Lass *et al.* (2001, Fig. 7C) reported day/night differences in gut fullness determined from the number of copepod mandibles in the gut. The krill contained 39% more mandibles at night and the standard deviation of the gut fullness was ≈25% of the mean. To detect a similar difference with a power = 0.8 required 17 replicates from each of day and night (df = 32) in our study. For the faecal pellet production, we used the clearance rates in Calbet *et al.* (1999, Fig. 1) for *C. typicus*, which were 75% higher at night and with a standard deviation 40% of the mean. With a standard deviation = 40% and a predicted difference of 50% between day and night, a design with 11 replicates from each of day and night (df = 20) was required for a power = 0.8. Since it was not possible to run all replicates in one experiment, the entire experiments were repeated three times in summer and in autumn.

Pilot experiments—correction factors for data analysis

The krill *M. norvegica* may feed on small-sized phytoplankton and microzooplankton cells (Agersted and Nielsen, 2016), as well as on detritus and sediments (Youngbluth *et al.*, 1989; Lass *et al.*, 2001). Therefore, pilot experiments were conducted to account for any potential effect of krill on *O. marina* concentration and/or copepod faecal pellet accumulation in the incubations.

To check for krill grazing on *O. marina*, 11 bottles of 4 L were filled with acclimatized filtered seawater and adjusted to 1 ppm of *O. marina* following the same methodology as in the main experiments. Among the 11 bottles, three bottles were used as initial bottles (only *O. marina*), four as control bottles (only *O. marina*) and four as experimental bottles (*O. marina* and one krill). The organisms were added to the bottles as described in the previous section. Control and experimental bottles were then incubated for 10 h under the same conditions as in the main experiments, and initial and final *O. marina* concentrations were measured with a Coulter counter.

Two incubations were carried out to determine krill grazing on copepod faecal pellets. Six bottles (first incubation) or 10 bottles (second incubation) of 4 L were filled with acclimatized filtered seawater. Half of the bottles served as control bottles (only faecal pellets) and the other half as experimental bottles (faecal pellets and one krill). Faecal pellets were collected from copepod tanks by siphoning the tank bottoms and removing copepods with a 200-μm mesh. The faecal pellet concentration was estimated by counting three subsamples, and then aliquots containing around 350 faecal pellets were added to each bottle. Krill were transferred to the experimental bottles using an aquarium net. The bottles were then incubated for 10 h in the same conditions as in the main experiments. At the end of the incubations, the krill and the faecal pellets were collected using a 20-µm mesh. The faecal pellets were fixed in acidic Lugol's solution for counting and size determination. A total of 60 faecal pellets per treatment (i.e. with and without krill) were photographed, and length and width measurements were conducted with ImageJ (Schneider *et al.*, 2012).

Data analysis

The feeding activity of copepods was estimated based on their faecal pellet production rates (Nejstgaard *et al.*, 2001; Besiktepe and Dam, 2002). The average pellet volumes were calculated assuming an ellipsoidal shape. Gut evacuation times of copepods (20 min at 14°C, Irigoien (1998)) were subtracted from incubation times because copepod guts were empty before incubations. In the experimental bottles where krill actively predated on copepods, the average number of copepods during incubation was calculated assuming an exponential decrease of copepod abundance following the equations in Frost (1972).

The pilot experiments showed that in 10-h incubations the *O. marina* concentrations in the bottles did not change regardless of the absence or the presence of krill (two-tailed Student's t-tests, $P > 0.05$). However, krill removed 15% of copepod faecal pellets during incubations (randomized block design (RBD) analysis of variance (ANOVA), $F(1,13) = 10.76$, $P < 0.01$). Thus, faecal pellet production rates of copepods were corrected assuming a pellet removal by krill of 1.5% per hour.

After data correction, RBD ANOVA tests with experiment as block factor were conducted to check for significant effects of the factors day/night and absence/presence of predator (krill) on copepod pellet production rates. RBD ANOVAs were applied to each set of experiments with the same photoperiod and prey concentration (i.e. experiments in summer at high food, and experiments in autumn at high food, Table I). For the only experiment at low food availability (1 ppm), a two-way ANOVA was used instead. Additionally, a two-way ANOVA was conducted to check for significant differences between seasonal photoperiod (16:8 h *vs* 10:14 h) and prey availability (high *vs* low) in the magnitude of copepod feeding rhythms (i.e. night/day ratios of pellet production rates). Finally, a two-way ANOVA was applied to krill predation rates to test significant differences between day and night, and between seasons (i.e. photoperiod). All datasets passed normality and homoscedasticity assumptions according to Shapiro–Wilk and Brown-Forsythe tests, respectively.

d) Results

The krill *Meganyctiphanes norvegica* was actively feeding on copepods in our experiments. The average predation rates ranged 0.1–0.6 cop krill^{-1} h^{-1} and no significant differences were detected between day and night, or between seasons (two-way ANOVA, F(1,54) = 1.51 and $P > 0.05$ for day/night, F(1,54) = 3.61 and $P > 0.05$ for photoperiod; Fig. 1). The interaction between factors was also not significant (two-way ANOVA, F(1,54) = 1.21, $P > 0.05$).

Table II shows the sizes of copepods and prey, as well as the faecal pellet production rates of copepods and the pellet volumes in the incubations. All the krill specimens had a body length of ca. 40 mm. The pellet production rates of copepods were significantly higher at night in all the experiments (summer at high food: RBD ANOVA, F(1,50) = 70.63, $P < 0.001$; autumn at high food: RBD ANOVA, F(1,27) = 226.82, $P < 0.001$; autumn at low food: two-way ANOVA, F(1,13) = 495.75, $P < 0.001$; Fig. 2). The presence of predator (krill) did not have any significant effect on pellet production rates in any of the experiments at high food (summer: RBD ANOVA, F(1,50) = 3.15, $P > 0.05$; autumn: RBD ANOVA, F(1,27) = 0.04, $P > 0.05$; Fig. 2), but had a significant effect in the experiment on 25 October at low food (two-way ANOVA, F(1,13) = 13.63, $P < 0.01$; Fig. 2). However, in this last case krill caused a decrease of only 12% in the daily production of faecal pellets by copepods. No significant interactions between the factors day/night and absence/presence of predator were found (summer at high food: RBD ANOVA, F(1,50) = 0.91, $P > 0.05$; autumn at high food: RBD ANOVA, F(1,27) = 0.73, $P > 0.05$; autumn at low food: two-way ANOVA, F(1,13) = 1.19, $P > 0.05$).

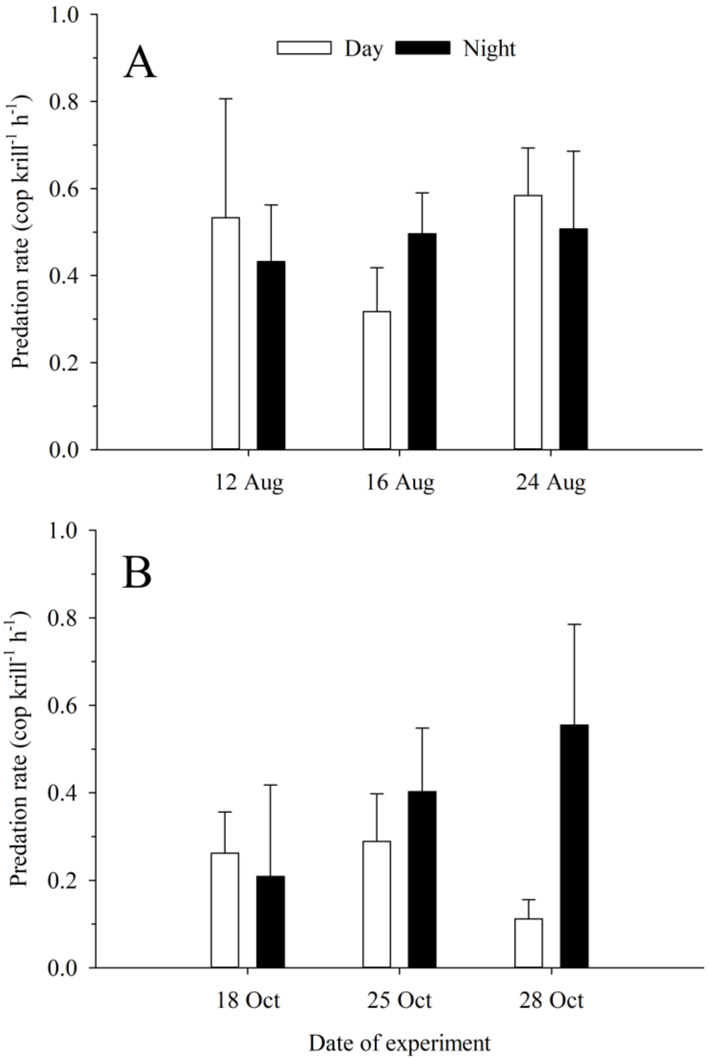

Fig. 1. Predation rates of the krill Meganyctiphanes norvegica (mean±SE, n = 4–6) on the copepod Centropages typicus in experiments conducted in (A) summer (16 h:8 h day: night cycle) and (B) autumn (10 h:14 h day: night cycle).

The intensity of copepod feeding rhythms (i.e. night/day ratios of pellet production rates) were significantly different between seasonal photoperiods (two-way ANOVA, F(1,9) = 29.97, $P < 0.001$) and between food conditions (two-way ANOVA, F(1,9) = 62.58, $P < 0.001$). The night/day ratios of pellet production were 1.2–1.4 in summer at high prey concentration, 1.6–1.8 in autumn at high prey concentration, and 2.3–2.4 in autumn at low prey concentration (Fig. 2).

Table II: Sizes of copepods (Centropages typicus) and prey (Oxyrrhis marina), faecal pellet production rates of copepods, and pellet volumes in incubations without predator ("Control") and with the predator Meganyctiphanes norvegica ("Predator"). Mean ± SE are shown. ESD: equivalent spherical diameter. n.d.: not determined.

				Control		Predator	
Date of experiment	Time period	Copepod size (µm)	Prey size (ESD, µm^3)	Pellet production (pellets cop^{-1} h^{-1})	Pellet volume (µm^3)	Pellet production (pellets cop^{-1} h^{-1})	Pellet volume (µm^3)
12 Aug	Day	n.d.	16.2 ± 0.05	1.5 ± 0.10	183462 ± 16954	1.5 ± 0.07	191441 ± 10065
	Night	n.d.	16.0 ± 0.08	1.8 ± 0.18	203021 ± 12497	1.8 ± 0.07	223268 ± 10884
16 Aug	Day	1182 ± 8.4	17.6 ± 0.03	1.3 ± 0.04	170313 ± 12730	1.0 ± 0.03	171732 ± 12338
	Night	1168 ± 8.6	16.6 ± 0.01	1.3 ± 0.06	199182 ± 8950	1.4 ± 0.07	189389 ± 8527
24 Aug	Day	1164 ± 14.3	16.5 ± 0.01	1.9 ± 0.04	288239 ± 11924	1.8 ± 0.06	268105 ± 10945
	Night	1186 ± 10.3	16.4 ± 0.03	2.1 ± 0.05	298799 ± 16467	1.9 ± 0.06	311400 ± 22596
18 Oct	Day	1256 ± 10.7	19.2 ± 0.03	1.9 ± 0.11	289399 ± 12370	1.9 ± 0.16	305818 ± 13861
	Night	1225 ± 13.8	18.1 ± 0.02	2.2 ± 0.09	400043 ± 22237	2.3 ± 0.03	428394 ± 24822
25 Oct	Day	1203 ± 10.9	20.4 ± 0.04	1.2 ± 0.04	220836 ± 13482	1.2 ± 0.06	189468 ± 10139
	Night	1205 ± 14.6	19.3 ± 0.08	1.8 ± 0.07	336411 ± 19286	1.6 ± 0.03	331894 ± 19619
28 Oct	Day	1203 ± 10.6	17.4 ± 0.04	1.7 ± 0.08	290791 ± 18121	1.5 ± 0.04	303520 ± 22506
	Night	1212 ± 14.5	17.0 ± 0.02	1.8 ± 0.05	443893 ± 24626	1.9 ± 0.06	412872 ± 22900

e) Discussion

Previous studies linked feeding rhythms of wild copepods to nocturnal forays into food-enriched upper layers during diel vertical migrations (Baars and Oosterhuis, 1984; Simard *et al.*, 1985; Besiktepe *et al.*, 2005). In our experiments, given the bottle sizes, we did not expect any relevant light-induced spatial heterogeneity in the distribution of the copepod prey *Oxyrrhis marina* (see Methods section) that could not be overcome by the swimming activity and the prey detection capability of *Centropages typicus* (Tiselius and Jonsson, 1990; Bundy *et al.*, 1993; Gonçalves and Kiørboe, 2015). Therefore, our study suggests that the rhythmic feeding behaviour of the copepod *C. typicus* might have an adaptive significance itself, which seems independent of migratory behaviour or changing food conditions

(Head *et al.*, 1985; Durbin *et al.*, 1990). As evidenced in our experiments, such rhythms do not necessarily imply the complete cessation of feeding during the daytime (*sensu* Dagg *et al.* (1998)), but a lower foraging activity during daylight hours (Atkinson *et al.*, 1996; Olivares *et al.*, 2020).

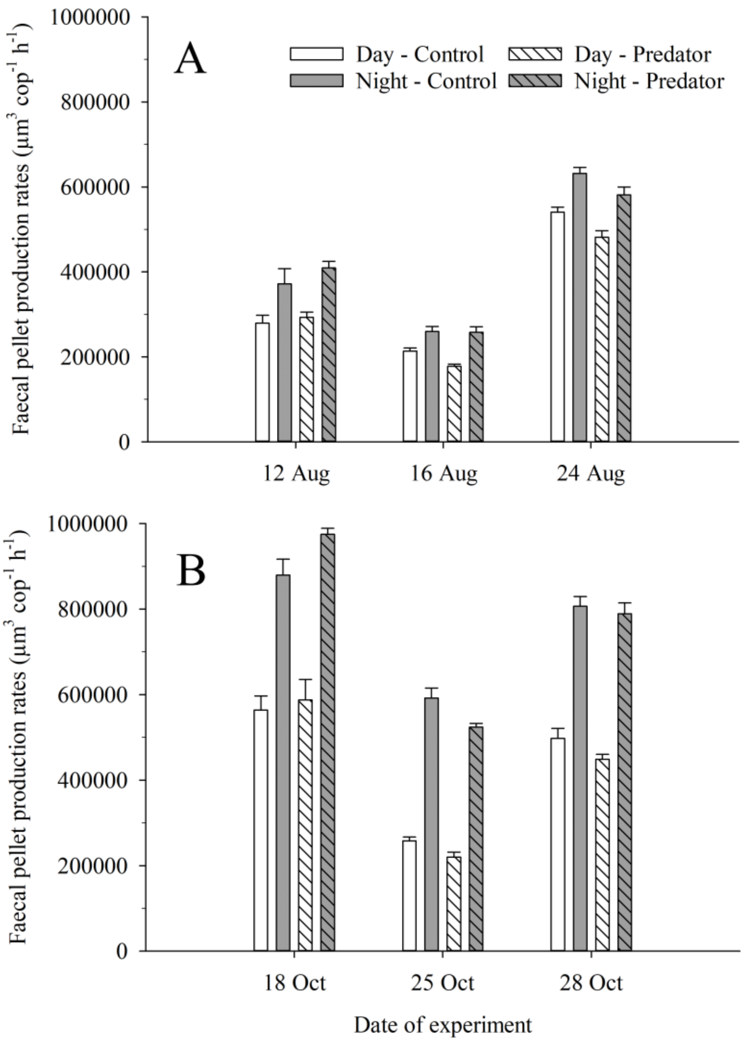

Fig. 2. Day and night faecal pellet production rates (mean±SE, n = 4–6) of Centropages typicus feeding on Oxyrrhis marina in the absence of predator ("Control") and in the presence of the predator Meganyctiphanes norvegica ("Predator"). Data from experiments in (A) summer (16 h:8 h day: night cycle) and (B) autumn (10 h:14 h day: night cycle) are shown. Notice that O. marina concentrations were 1 ppm on 25 October, and 5.5–7.0 ppm in the other experiments.

The diel activity patterns of marine copepods can change upon the presence of predators (Ohman, 1988; Bollens and Frost, 1989; Bollens and Stearns, 1992). The krill *Meganyctiphanes norvegica* exert an important predatory pressure on copepod populations in the Northeast Atlantic (Beyer, 1992; Båmstedt and Karlson, 1998; Onsrud and Kaartvedt, 1998) and can affect their vertical migration patterns (Tarling *et al.*, 2002). Because the krill densities in our experiments were higher than typical average abundances in nature (Onsrud and Kaartvedt, 1998; Tarling *et al.*, 1998), we would expect the predator-induced responses of copepods to have been maximized. Still, we did not detect any large effect of krill presence on the feeding behaviour of copepods, either in diel feeding patterns or daily food intake. In all the cases the presence of krill barely affected the mean daily productions of copepod faecal pellets (from -8.7 to 12.2%), and such small differences proved to be statistically significant only in one out of the six experiments. Given the low variability among replicates in the faecal pellet production rates (median of coefficients of variation: 7.8%), any undetected effect of krill on copepod feeding activity was small. Thus, the non-lethal direct effects of predators on the feeding activity of marine copepods, including diel feeding rhythms, could be limited and/or depend on type of predator (Ohman, 1990).

In our study, *M. norvegica* showed predation rates that were highly variable among individuals. Such a flexible feeding behaviour has been previously observed in other experimental studies with *M. norvegica* predating on copepods (McClatchie, 1985; Båmstedt and Karlson, 1998; Agersted and Nielsen, 2016). We did not detect differences between day and night or between seasons in krill predation rates, but given the high variability of predation rates and the sample size in our experiments, we cannot discard that certain diel or seasonal differences in the feeding activity of krill could have been unnoticed (Torgersen, 2001). Our design was based on a power = 0.8 and standard deviations of 25–40% of the mean, but we observed a much higher variability than anticipated (median = 94% of the mean). It is also possible that the diurnal predation rates of *M. norvegica* could have been enhanced if copepods had been feeding on a more pigmented prey (e.g. phytoplankton) that increases copepod susceptibility to visual predation (Juhl *et al.*, 1996; Tsuda *et al.*, 1998), instead of an heterotrophic, not pigmented protist.

Still, Abrahamsen *et al.* (2010) reported that *M. norvegica* might rely more on hydromechanical signals than vision to detect active prey like copepods. Actually, *M. norvegica* conduct diel vertical migrations (Onsrud and Kaartvedt, 1998; Onsrud *et al.*, 2004) and most encounters between *M. norvegica* and copepods might take place at night when visual predation is limited. Particularly in the Gullmar Fjord, populations of *M. norvegica* remain deeper during the daytime and ascend to layers above 30 m only at night (Spicer and Strömberg, 2002). Thus, copepods may find more advantageous to modify their diel antipredator feeding behaviour upon the presence of other visual predators like fish that occur in upper, more illuminated layers during daylight hours (Øresland and André, 2008).

We found that seasonal photoperiod and prey availability had a significant influence on the diel feeding behaviour of copepods. The feeding rhythms were less pronounced in summer (16 h of light) than in autumn (10 h light). The diel rhythms of marine copepods are usually flexible over seasons (Båmstedt, 1984; Williams and Conway, 1984; Frost, 1988; Durbin *et al.*, 1995). Frost (1988) suggested that such variations between seasons are independent of prey availability, metabolic balance or thermal stratification, but driven by predation risk. In this regard, the seasonal photoperiod defines the relative time that copepods are exposed to a higher visual predation risk. As periods of higher predation vulnerability become relatively longer, copepods might diminish their antipredator behaviour to optimize the trade-off between eating and not being eaten (Lima and Bednekoff, 1999). Clearly, copepods must lower the intensity of their feeding rhythms when safe periods (i.e. dark periods) are too short for feeding to meet metabolic demands.

Regarding prey availability, the hunger/satiation hypothesis affirms that higher food availability normally results in larger amplitudes of vertical migration (Huntley and Brooks, 1982; Verheye and Field, 1992), which would enhance copepod feeding rhythms if food conditions in upper layers are better (Simard *et al.*, 1985; Peterson *et al.*, 1990; Besiktepe *et al.*, 2005). We found that the diel rhythms of *C. typicus* in the laboratory were more intense at low food concentrations. Low-food conditions decrease encounter rates with prey and copepods must swim for longer times and cover larger distances to feed (Saiz *et al.*, 1992), thus increasing their risk of being detected by predators (Uttieri *et al.*, 2013; Kiørboe *et al.*, 2014). Under these conditions, copepods would instead enhance their nocturnal feeding activity because foraging during the daylight hours would be too risky for them. We cannot strictly test this hypothesis since we only conducted one low-food experiment, but our findings are in agreement with those of Hassett and Blades-Eckelbarger (1995), who found that the diel feeding cycles of *Acartia tonsa* were more pronounced in the low-food treatment. In contrast, Calbet *et al.* (1999) reported that at lower food concentrations the diel feeding rhythms of *C. typicus* remained invariable and those of *A. grani* even vanished. Hence, the effect of food availability on copepod nocturnal feeding remains unclear and might be species-specific and/or depend on environmentally determined previous life history.

f) Conclusions

Our study is one of the few works that addressed direct effects of predators on copepod feeding rhythms using freely swimming predators instead of predator exudates. Still, we did not detect any large effect of the predator *Meganyctiphanes norvegica* on the diel feeding behaviour of the marine calanoid *Centropages typicus*, whereas effects of other factors such as seasonal photoperiod and prey availability emerged. Therefore, the non-lethal direct effects of predators on the feeding activity of marine copepods might not be as relevant as in freshwater zooplankton, and it will require further effort to assess their role in plankton trophic interactions in marine systems.

g) Acknowledgements

We would like to thank Ursula Schwarz for assisting with zooplankton sampling, Bengt Lundve for technical support and Kirti Ramesh, Ana Lopes and Theresa Haller for helping with phytoplankton culture maintenance. This study is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

h) Funding

The research leading to these results received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730984, ASSEMBLE Plus project. The open access publication fee was covered by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). This study was framed within the project FERMI (CGL2014–59227-R, MINECO/AEI/FEDER, UE), and M.O. was supported financially by an FPU grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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4.4. The neritic marine copepod *Centropages typicus* does not suffer physiological costs from diel temperature fluctuations involved in vertical migration

Reference:
Olivares, M.; Calbet, A.; Saiz, E. (In prep.). The neritic marine copepod *Centropages typicus* does not suffer physiological costs from diel temperature fluctuations involved in vertical migration.

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a) Abstract

Migrant zooplankton usually face fluctuating environmental conditions through vertical migration. In our study, we investigated the physiological costs of diel temperature fluctuations of vertical migration in the neritic marine copepod *Centropages typicus*, and checked whether the direct effects of fluctuating temperature on copepod performance may differ from those of average temperature conditions (Jensen's inequality). We exposed this copepod species to four temperature regimes reflecting different migratory patterns (including fluctuating temperature caused by vertical migration), and determined the rates of egestion, egg production, and respiration. We found that the physiological performance of *C. typicus* was not significantly different between fluctuating and average temperature conditions, thus indicating that differences between varying and average temperature effects might not emerge in migrant zooplankton exposed to thermal variations of moderate magnitude (5 °C). The copepods exposed to fluctuating temperature mimicking vertical migration showed a better performance (e.g. higher egg production) than those exposed to constant depth (cold) conditions; however, no significant differences were detected when compared to constant surface (warm) conditions. Our study indicates that neritic marine copepods experiencing daily moderate temperature fluctuations through vertical migration may not suffer physiological costs associated to this behaviour.

In this sense, these animals might have physiological mechanisms to compensate diel temperature fluctuations, which would represent a particularly relevant preadaptation to cope with the increased thermal variability predicted in future climates.

b) Introduction

Zooplankton represent a vital trophic link between primary producers and fish in aquatic ecosystems (Turner, 2004; Sommer & Sommer, 2006). Many species of zooplankton conduct diel vertical migrations, frequently staying in upper waters at night and moving to deeper, darker layers during the daytime (Haney, 1988). This migratory behaviour results in adaptive benefits such as a lower mortality risk from visual predation, or the protection against damaging UV radiation during daylight hours (Bollens & Frost, 1989, 1991; Williamson et al., 2011). However, migrant zooplankton typically face different conditions of temperature and food availability through the water column that might affect their fitness (Geller, 1986; Loose & Dawidowicz, 1994; Pearre, 2003). McLaren (1963) and Enright (1977) initially suggested that feeding in upper, warmer layers at night and resting in deeper, colder waters during the daytime could provide an energy bonus to invest in growth and reproduction. Experimental studies, however, demonstrated that fluctuating environmental conditions experienced by diel migrators (mostly *Daphnia*) usually imply detrimental metabolic costs compared to non-migrating conditions in upper layers (Swift, 1976; Orcutt & Porter, 1983; Stich & Lampert, 1984; Lampert et al., 1988; Loose & Dawidowicz, 1994). In this respect, migrant zooplankton might be better adapted to cope with fluctuating conditions of vertical migration than non-migrants, thus optimizing the trade-offs derived from their migratory behaviour (Stich & Lampert, 1984).

Changes in temperature during vertical migration could have even a more significant impact than varying food conditions on the life history traits of zooplankton (Loose & Dawidowicz, 1994; Winder et al., 2003). Generally, zooplankton exposed to diel temperature fluctuations show a physiological performance that is intermediate between the performances at the lower and upper thermal limits (Vuorinen, 1987; Reichwaldt et al., 2005). The effects of fluctuating temperature on animals, however, might not be necessarily inferred from the study of average temperature effects according to the mathematical property of nonlinear averaging known as *Jensen's inequality* (Ruel & Ayres, 1999). The differences between fluctuating and average temperature effects predicted by Jensen's inequality have been indeed confirmed for a wide variety of physiological processes in animals, particularly in insects (Colinet et al., 2015), but still remain poorly documented in aquatic organisms facing wide temperature fluctuations on a daily basis, such as migrant zooplankton. In this regard, Orcutt and Porter (1983) found that the life history parameters of *Daphnia parvula* could be positively affected by fluctuating temperature of vertical migration in comparison to constant mean temperature conditions. Lock and McLaren (1970), however, reported that the female lengths of the copepod *Pseudocalanus minutus* did not show any significant difference between varying and average temperature regimes. The recent increasing awareness of Jensen's inequality among biologists has certainly improved our capability to determine the physiological performance of organisms under natural conditions of short-term temperature fluctuations (Denny, 2017; Morash et al., 2018). Despite the growing number of studies, most of the recent literature about the effects of fluctuating temperature on zooplankton has focused on freshwater cladocerans (Mikulski et al., 2017; Isanta Navarro et al., 2019; Hahn & von Elert, 2020), whereas such effects remain largely unexplored in other important zooplankton groups like copepods (Zhou & Sun, 2017).

Copepods are among the most abundant mesozooplankton worldwide, particularly in pelagic systems (Longhurst, 1985; Boxshall & Defaye, 2008). Many copepod species face temperature fluctuations through vertical migration in both freshwater and marine environments (Roe, 1984; Geller, 1986; Atkinson et al., 1992). It is well known that temperature can affect copepod fitness regarding their rates of growth and development (Vidal, 1980; Huntley & Lopez, 1992), feeding (Kiørboe et al., 1982; Durbin & Durbin, 1992), egg production (Hirche et al., 1997; Koski & Kuosa, 1999), and oxygen consumption (Hirche, 1987; Ikeda et al., 2001). Considering the current lack of records, the study of the effects of temperature fluctuations on copepod performance is essential knowledge to improve our capability to estimate energy fluxes within aquatic food webs, as well as provide insights about the susceptibility of copepods to future climate change scenarios (Vasseur et al., 2014).

In our study, we aimed to determine the physiological costs of diel temperature fluctuations involved in vertical migration in the neritic marine copepod *Centropages typicus*, and examine the different effects that might emerge between varying and average temperature conditions (Jensen's inequality). We exposed this copepod species to four temperature regimes reflecting different migratory patterns, and analysed functional traits related to feeding, reproduction and basal metabolism. The range of temperatures used corresponded to those experienced by this species in the NW Mediterranean throughout the extent of its vertical migration (Alcaraz, 1985; Saiz et al., 1992, 1999). Additionally, we checked whether the physiological performance of copepods under fluctuating temperature conditions could be predicted accurately from their performance in the upper and lower thermal limits.

c) Materials and methods

Prey and copepod cultures

The dinoflagellate *Oxyrrhis marina* was used as copepod food in copepod cultures and experiments. *O. marina* was grown in 0.1-μm filtered seawater fed with the algae *Rhodomonas salina*. The stock culture of *R. salina* was grown in f/2 medium (Guillard, 1983). The *O. marina* cultures were not supplied with food for 48 h before experiments to ensure the absence of *R. salina* cells during incubations. All the *O. marina* and *R. salina* cultures were kept in a temperature-controlled room at 19±0.5 °C and a 10 h: 14 h light: dark cycle.

Eggs from the calanoid copepod *C. typicus* were obtained from a copepod stock culture kept at the Institut de Ciències del Mar (ICM, CSIC). This copepod line was collected in waters off Barcelona and isolated in May 2017. *C. typicus* may reach abundances of several hundreds of individuals per cubic meter in coastal waters of the Mediterranean Sea, the North Sea, and different areas of the East and North Atlantic Ocean (Ianora & Buttino, 1990; Calbet et al., 2001; Halsband-Lenk et al., 2002; Bonnet et al., 2007). Around 25000 eggs were siphoned out from the tank bottom of the copepod stock culture and placed in a new tank with 15 L of 0.1-µm filtered seawater. The new copepod cohort was fed with *O. marina* and kept at the same temperature and photoperiod conditions as specified for the prey cultures. During the development process of the cohort, the volume of the copepod culture was increased from 15 L (nauplii) to 20-28 L (copepodites) and 32 L (adults). At the moment of the experiments, there were about 10000-12000 adults (1:1 sex ratio) in the tank.

Copepod acclimation to temperature regimes before experiments

Four open 72-L baths containing 33 L of filtered seawater were set at different temperature regimes using Teco water temperature conditioners (models TK2000 and TK500). The four temperature regimes were selected according to the typical temperatures that copepods (*C. typicus*) may experience in the Western Mediterranean in spring (Alcaraz, 1985; Saiz et al., 1999). The temperature regimes included one treatment with fluctuating temperature that simulated diel vertical migration (DVM, 10 h at 14 °C during the daytime, and 14 h at 19 °C at night), and three treatments with constant temperatures: 14 °C (equivalent to 24 h at depth), 19 °C (equivalent to 24 h at the surface), and 17 °C (average DVM conditions considering the actual time spent at each temperature).

Four 4-L Nalgene bottles with filtered seawater were placed in each bath. In the case of fluctuating temperature treatment (DVM), the temperature of the Teco temperature-conditioner was manually switched to 14 and 19 °C at 8:30 and 18:30, respectively. Previous checks showed that the 5 °C temperature shifts in the temperature of the Nalgene bottle contents were fully reflected within a 2-h timeframe, with most of the variation (4 °C) occurring within the first hour. Adult copepods were collected from the main copepod tank with a 335-µm mesh sieve, and 160 individuals (1:1 sex ratio) were added to each Nalgene bottle. The copepods were acclimated to the different temperature regimes and fed *ad libitum* with *O. marina* (>5ppm, van Someren Gréve *et al.*, (2017)) for 5-7 days before the experiments (Hirche et al., 1997). The prey concentrations in the Nalgene bottles were controlled daily by removing 2 L from each bottle and adding new water and *O. marina* to reach concentrations of 11-11.5 ppm. The *O. marina* concentrations from stock cultures and Nalgene bottles were determined with a Multisizer III Coulter counter. During the acclimation period, copepods were under a 10 h: 14 h light: dark cycle.

Experimental set-up and sample processing

Two consecutive experiments were conducted on 7 and 9 July 2019 at Institut de Ciències del Mar (ICM, CSIC) using copepods that were temperature-acclimated for 5 and 7 days, respectively. The experiments consisted of 24-h incubations to determine the carbon and nitrogen contents as well as the rates of feeding, egg production, and respiration of *C. typicus* at the four temperature regimes specified before (i.e. DVM, 14 °C, 17 °C, 19 °C). Before the incubations, the copepods were collected from the Nalgene bottles with a 335-µm mesh sieve and placed in 250-mL bottles with temperature-conditioned filtered seawater for ca. 2 h until been transferred to the experimental bottles.

The feeding activity of copepods was assessed according to their egestion (defecation) rates (Nejstgaard et al., 2001; Besiktepe & Dam, 2002). The rates of egestion and egg production were analysed in the same incubation bottles (620 mL), whereas the respiration rates were determined in separate bottles (67 mL). For egestion and egg production rates, a suspension of *O. marina* (ESD: 18.7±0.8 µm) at 8.5 ppm (ca. 2200-2800 cells mL^{-1}) was prepared using the Coulter Counter. Sixteen 620-mL Pyrex bottles (4 bottles per temperature treatment) were filled with the prey suspension, and eight females of *C. typicus* were added to each bottle using a wide-mouth glass pipette. The bottles were then incubated for 24 h in the corresponding temperature treatments. At the end of the incubations, the bottle contents were sieved through a 200-µm mesh to collect copepods, and then through a 20-µm mesh to collect eggs and faecal pellets. Copepod survival was checked under a stereomicroscope, and the number of dead animals was noted (only one dead copepod in two out of thirty-two bottles considering both experiments). Copepods, eggs and faecal pellets were then fixed in either 4% formalin solution (copepods) or 2% acidic Lugol's solution (eggs and faecal pellets) for counting and size determination. The faecal pellets and eggs were counted using a stereomicroscope. Photos of 15-20 copepod females and 180-200 faecal pellets were taken for each temperature treatment using an inverted microscope. The prosome length of copepods, and the length and width of faecal pellets were measured with the software ImageJ (Schneider et al., 2012). An ellipsoidal shape was assumed to calculate average pellet volumes.

The respiration rates of copepods were estimated based on the decrease of water oxygen saturation in the incubation bottles. Oxygen concentration in the water was measured with a PreSens 4-channel oxygen meter fitted with PSt3 probes. Temperature-conditioned filtered seawater at 89-98% oxygen saturation was used to fill thirty-two 67-mL incubation bottles (eight bottles per treatment). Half of the bottles (four per treatment) served as control bottles (only water), and the other half as experimental bottles (water with copepods). Twelve females of *C. typicus* were pipetted into each experimental bottle and all the bottles were incubated for 24 h. The copepod densities in the bottles should not affect the respiration rates of copepods (Nival et al., 1972; Svetlichny et al., 2012). After the incubations, the oxygen saturation in the water was measured for each bottle. The final oxygen saturations in the experimental bottles (i.e. bottles with copepods) were on average 84% and never fell below 77%. The copepods from the bottles were then sieved with a 200-μm mesh and their survival was checked under a stereomicroscope (one dead copepod in three out of thirty-two bottles in the two experiments). Subsequently, the copepods were fixed in formalin (4 %) for counting, photographing and size measurements.

Finally, the carbon and nitrogen contents of copepods were determined using twenty-one pre-combusted GF/C filters in each experiment: one filter served as the filter blank (only filter), four filters as the water blanks (filter with water, one replicate per temperature treatment), and sixteen filters as the filters with copepods (ten females per filter, four replicates per temperature treatment). The filters were kept in Eppendorf vials, let dry at 60 °C for 48 h, and the carbon and nitrogen contents were determined with a FlashEA1112 Elemental Analyser.

Data analysis and statistics

The Q_{10} coefficients for the rates of egestion, egg production, and respiration of *C. typicus* were estimated based on the rates measured in the three temperature-fixed treatments (i.e., 14, 17, and 19 °C). The Q_{10} coefficients were calculated through the equation $Q_{10}=e^{(\frac{10E_a}{RT^2})}$ (Raven & Geider, 1988), where *Ea* is the activation energy (J mol^{-1}), *R* is the constant gas (8.3145 J K^{-1} mol^{-1}), and *T* is the mean temperature (K) of the temperature range used to determine the Q_{10} coefficient. The activation energies of the different processes were obtained following the steps specified in Alcaraz *et al.* (2013).

Statistical tests were performed using the software IBM SPSS Statistics 26. Randomized block design (RBD) ANOVA tests (carbon contents, nitrogen contents, and molar C:N ratios of copepods), and a RBD MANOVA test (rates of egestion, egg production, and respiration) with experiment as block factor were applied to detect significant differences among temperature treatments. When temperature had a significant effect, Dunnett's tests were run to check for significant differences between DVM (i.e. treatment with diel temperature fluctuations) and the other treatments with fixed temperature. All the datasets met the assumptions of normality and homogeneity of variance according to Shapiro-Wilk and Levene's tests, respectively.

Finally, the rates of egestion, egg production, and respiration of copepods in the treatments with fixed temperature of 14 and 19 °C were used to predict the expected copepod physiological performance in the DVM treatment (i.e. diel fluctuations between 14 and 19 °C). For that purpose, the rates at 14 °C and those at 19 °C were weighted, respectively, by the time lengths of the day and night periods (10 and 14 h, respectively). Z-tests were then run to check for significant differences between the predicted and the observed rates of egestion, egg production, and respiration of copepods in DVM conditions.

d) Results

Table 1 shows the prosome lengths, the carbon and nitrogen contents, and the molar C:N ratios of *C. typicus* in the different temperature treatments. As expected, the prosome lengths were very similar in all the treatments (overall mean: 1019±3.5 μm), as the copepods originated from the same cohort and developed until adults under the same conditions. The average carbon contents seemed to decrease with temperature from 7.2±0.23 μg C cop^{-1} at 14 °C to 6.4±0.36 μg C cop^{-1} at 19 °C, although no significant differences were detected among temperature treatments (RBD ANOVA, F(3, 27)=1.326, $p>0.05$). The average nitrogen contents showed significant differences among treatments (RBD ANOVA, F(3, 27)=3.535, $p<0.05$) with higher values at 14 °C (ca. 2.0 μg N cop^{-1}) than in the other treatments (ca. 1.7 μg N cop^{-1}). The nitrogen contents were significantly different between DVM (i.e. fluctuating temperature of vertical migration) and 14 °C (Dunnett's test, $p<0.05$), but not between DVM and 17 or 19 °C (Dunnett's tests, $p>0.05$). The average molar C:N ratios of copepods ranged 4.2-4.6 and showed significant differences among temperatures (RBD ANOVA, F(3, 27)=7.605, $p<0.001$). The C:N ratios in DVM (4.6±0.07) were significantly higher than at 14 °C (4.2±0.05) (Dunnett's test, $p<0.001$), but no significant differences were detected compared to 17 or 19 °C (Dunnett's tests, $p>0.05$).

Table 1. Prosome lengths, carbon (C) and nitrogen (N) contents, and molar C:N ratios of Centropages typicus in different temperature treatments. DVM: simulation of diel vertical migration (10 h at 14 °C and 14 h at 19 °C). Data from the two experiments were pooled. Means ± SE are provided. Significant differences with DVM are indicated with an asterisk (Dunnett's test, p<0.05 for N, p<0.001 for C:N ratio)

Temperature	Prosome length (μm)	C (μg)	N (μg)	C:N ratio
DVM	1024 ± 5.1	6.6 ± 0.35	1.7 ± 0.09	4.6 ± 0.07
14 °C	1019 ± 6.3	7.2 ± 0.23	2.0 ± 0.06*	4.2 ± 0.05*
17 °C	1018 ± 4.7	6.7 ± 0.31	1.7 ± 0.08	4.5 ± 0.04
19 °C	1014 ± 7.7	6.4 ± 0.36	1.7 ± 0.09	4.5 ± 0.06

The faecal pellet volumes, and the rates of egestion, egg production, and respiration of copepods in the experiments are shown in Table 2. The egestion rates were significantly different among temperature treatments (RBD MANOVA, F(3, 27)=2.960, $p<0.05$), and increased about 20% from 14 °C to 17-19 °C (Q_{10}=1.4) (Fig. 1a). However, no significant differences were detected between DVM and the other temperatures (Dunnett's tests, $p>0.05$) (Fig. 1a). The egg production rates showed significant differences among treatments (RBD MANOVA, F(3, 27)=18.723, $p<0.001$), increasing with temperature from 60±3.9 eggs cop^{-1} d^{-1} at 14 °C to 91±3.4 eggs cop^{-1} d^{-1} at 19 °C (Q_{10}=2.3) (Fig. 1b). The egg production rates in DVM (85±2.1 eggs cop^{-1} d^{-1}) were significantly higher than those at 14 °C (Dunnett's test, $p<0.001$), but not statistically different to those at 17 or 19 °C (Dunnett's tests, $p>0.05$) (Fig. 1b). The respiration rates also showed significant differences among temperature treatments (RBD MANOVA, F(3, 27)=9.445, $p<0.001$) and increased from 0.129±0.007 µmol O_2 cop^{-1} d^{-1} at 14 °C to 0.179±0.008 µmol O_2 cop^{-1} d^{-1} at 19 °C (Q_{10}=2.0) (Fig. 1c). The patterns of respiration rates were similar to those of egg production: the respiration rates in DVM (0.175±0.010 µmol O_2 cop^{-1} d^{-1}) were significantly higher than at 14 °C (Dunnett's test, $p<0.001$), but no statistical differences were detected compared to the rates at 17 or 19 °C (Dunnett's tests, $p>0.05$) (Fig. 1c). When considering the carbon contents of copepods (i.e. weight-specific rates), the dependence on temperature was stronger in all the physiological processes with Q_{10} coefficients of 1.8 for egestion, 2.9 for egg production, and 2.5 for respiration.

Table 2. Faecal pellet (FP) volumes, and rates of egestion, egg production, and respiration of Centropages typicus in different temperature treatments. DVM: simulation of diel vertical migration (10 h at 14 °C and 14 h at 19 °C). Data from the two experiments were pooled. Means ± SE are provided. Significant differences with DVM are indicated with an asterisk (Dunnett's tests, p<0.001)

Temperature treatment	FP volume (µm^3)	Egestion rate (FP cop^{-1} d^{-1})	Egestion rate (µm^3 cop^{-1} d^{-1})	Egg production rate (eggs cop^{-1} d^{-1})	Respiration rate (µmol O_2 cop^{-1} d^{-1})
DVM	124383 ± 5406	36 ± 1.3	4513995 ± 152369	85 ± 2.1	0.175 ± 0.010
14 °C	133014 ± 9033	32 ± 1.4	4255466 ± 245192	60 ± 3.9*	0.129 ± 0.007*
17 °C	133374 ± 5211	39 ± 2.5	5232905 ± 308786	77 ± 3.2	0.175 ± 0.005
19 °C	123448 ± 7733	41 ± 2.6	5004039 ± 317118	91 ± 3.4	0.179 ± 0.008

The predicted rates of egestion, egg production and respiration in DVM based on the rates observed at 14 and 19 °C and the times spent at each temperature were comparable to the actual rates observed in the DVM treatment (Table 3). In the case of egg production and respiration rates, the average predicted values were a bit lower than those observed, although such small differences were not statistically significant (Z-tests, p>0.05).

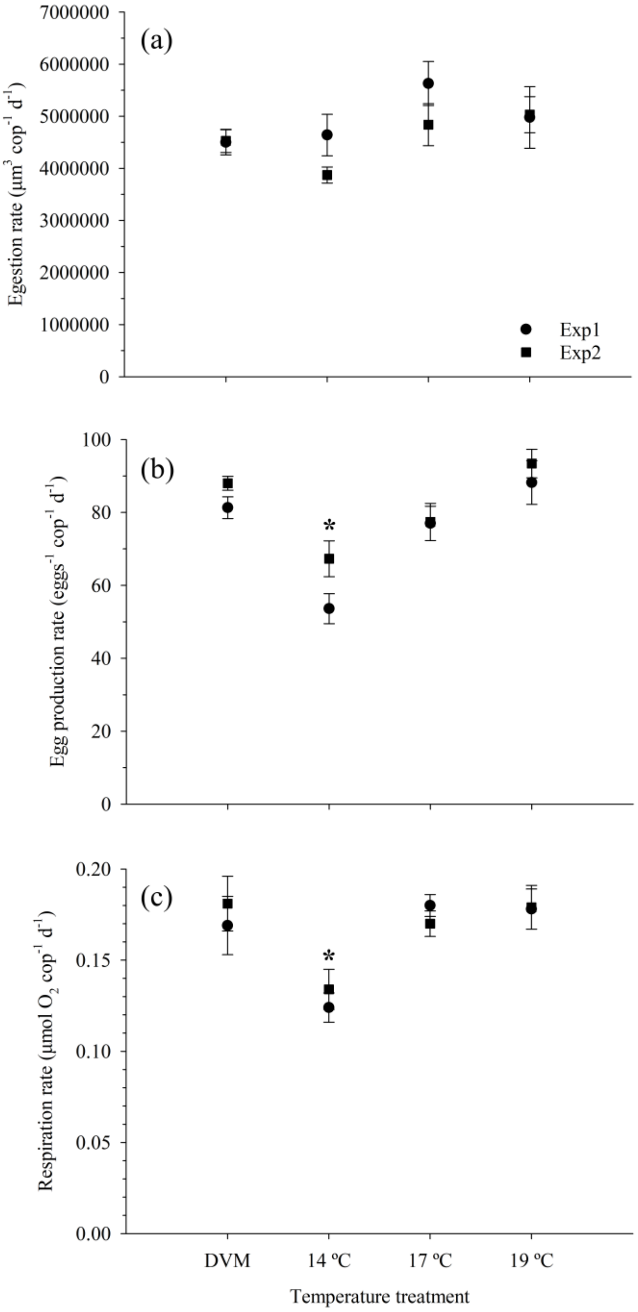

Fig. 1. Rates of egestion (a), egg production (b), and respiration (c) of Centropages typicus under different temperature treatments. DVM: simulation of diel vertical migration (10 h at 14 °C and 14 h at 19 °C). Means ± SE bars from the two experiments are shown. Asterisks indicate significant differences with DVM (Dunnett's tests, p<0.001)

Table 3. Observed and predicted rates of egestion, egg production and respiration of Centropages typicus under fluctuating temperature conditions of diel vertical migration. The observed values refer to measurements obtained in the DVM treatment (10 h at 14 °C and 14 h at 19 °C), and the predicted values are calculated according to the rates measured at 14 °C and 19 °C and the number of hours at each temperature in the DVM treatment. Data from the two experiments are shown. Means ± SE are provided. The SE of the predicted values were calculated following error propagation rules

Experiment	Estimate	Egestion rate (μm^3 cop^{-1} d^{-1})	Egg production rate (eggs cop^{-1} d^{-1})	Respiration rate (μmol O_2 cop^{-1} d^{-1})
Exp 1	Observed	4499994 ± 241660	81 ± 3.0	0.169 ± 0.016
	Predicted	4836966 ± 381999	74 ± 3.9	0.156 ± 0.007
Exp 2	Observed	4527997 ± 223187	88 ± 1.9	0.181 ± 0.015
	Predicted	4547302 ± 212647	82 ± 3.0	0.160 ± 0.009

e) Discussion

The temperature range used in our experiments (14-19 °C) represented the typical thermal conditions that the copepod *C. typicus* may experience during vertical migration in the north-western Mediterranean (Alcaraz, 1985; Saiz et al., 1999). The Q_{10} coefficients that we obtained for the rates of egg production and respiration (2.3 and 2.0, respectively) were similar to those reported in previous studies for this species (Fernández, 1978; Halsband-Lenk et al., 2002). In the case of the egestion rates, however, the dependence on temperature was lower than expected (Q_{10}=1.4). It is surprising the little effect found here, as there is an extensive bulk of literature about the significant correlation between temperature and rates of ingestion and gut evacuation in planktonic copepods (Fernández, 1978; Kiørboe et al., 1982; Dam & Peterson, 1988; Durbin & Durbin, 1992). In this sense, the egestion rates of copepods are documented as a good proxy for their ingestion rates (Nejstgaard et al., 2001; Besiktepe & Dam, 2002). However, temperature can also affect the absorption efficiency of copepods (i.e. the portion of ingested food that is digested and absorbed; Almeda *et al.* (2011)), likely affecting the degree of packaging of the non-absorbed food remains. Therefore, it appears that, unfortunately, the egestion rates of copepods might not be the best parameter to determine the temperature dependence of their feeding activity. For that reason, egestion rates are not further considered in the discussion, even though these results do not contradict any of our conclusions.

In our study, we did not detect differences between varying and average temperature effects on copepod performance predicted by Jensen's inequality, as the rates of egg production and respiration of *C. typicus* exposed to fluctuating temperature (10 h at 14 °C and 14 h at 19 °C) were similar to those at 17 °C. Even though Lock and McLaren (1970) also found no evidence of Jensen's inequality when analysing changes in the female lengths of *P. minutus* in the thermal range 8-12 °C, other studies with zooplankton indicate that different outcomes between varying and average temperature regimes may appear upon wider temperature fluctuations (Orcutt & Porter, 1983). The existing literature on this matter is still scarce, but it seems that the physiological performance of migrant zooplankton under temperature variations could be estimated through the study of average temperature effects as long as the amplitude of thermal variations is moderate. According to our results, average estimates based on the physiological performances at the upper and lower thermal limits and the times spent at each temperature would prove also suitable. Although migrant zooplankton may experience diel temperature fluctuations of relatively small amplitude (i.e. up to 5-6 °C) (Saiz & Alcaraz, 1990; Cooke et al., 2008; Almén et al., 2014), other diel migrators from both marine and freshwater environments face temperature changes of 10 °C or more when moving between layers (Loose & Dawidowicz, 1994; Svetlichny et al., 2000; Al-Mutairi & Landry, 2001). In that case, differences between fluctuating and average temperature effects might emerge, and estimating the temperature-dependent performance of migrant zooplankton through the study of fixed temperature effects may become more challenging.

In our experiments, the copepods exposed to the regime of temperature fluctuations experienced through diel vertical migration showed a better performance (e.g. higher egg production) than those at constant, deep-water temperature (14 °C), but no differences were detected compared to those kept at constant surface temperature (19 °C). Therefore, our results indicate that copepods do not necessarily suffer physiological costs from daily temperature fluctuations involved in vertical migration, at least those species like *C. typicus* that may experience a moderate range of temperature variation. In this respect, migrant zooplankton may have adaptive physiological mechanisms to compensate diel temperature fluctuations. Recent proteomic analyses indicate that migrant copepods experience distinct physiological shifts derived from their migratory behaviour (Maas et al., 2018). In our study, the carbon and nitrogen contents of copepods under temperature fluctuations did not differ from those at 17 or 19 °C, indicating that any temperature-induced adjustment in the copepod physiology might imply the expression of alternative protein variants, or the modulation of the protein environment (Clarke, 2003). In copepods of relatively small size such as *Centropages* spp., changes in the synthesis and accumulation of small amounts of triglycerides might also happen upon temperature variations (Båmstedt, 1986), although we think that such modifications in the lipid content would barely affect the copepod carbon: nitrogen ratios. In larger copepods with a greater capacity to accumulate lipid reserves (e.g. seasonal migrators), however, temperature-induced changes in the storage and composition of lipid compounds may play an important role to cope with temperature and pressure variations during vertical migration (Pond et al., 2014). In this regard, Zhou and Sun (2017) reported that diel temperature fluctuations could promote lipid accumulation in *Calanus sinicus*, and Hassett and Crockett (2009) found that *Calanus finmarchicus* was the only copepod among five species that showed changes in cholesterol levels across different temperatures.

Our study demonstrates that the temperature fluctuations involved in vertical migration might not imply metabolic disadvantages in migrant zooplankton, at least in marine copepods that experience temperature changes within a moderate range (5 °C). However, we did not consider diel changes in other environmental variables besides temperature that can also affect copepod performance. For instance, diel migrators may face important variations in food availability and oxygen saturation through the water column (Saiz et al., 1992; Atkinson et al., 1996; Saltzman & Wishner, 1997; Wishner et al., 1998). In this regard, previous studies showed that the copepods *C. typicus* and *Paracartia grani* were capable to deal successfully with short-term fluctuations in food intake (Davis & Alatalo, 1992; Calbet & Alcaraz, 1996; Olivares et al., 2020). The effects of changing temperature on zooplankton, however, can covariate with those of other environmental variables (Koussoroplis & Wacker, 2016), and the joint effects of varying temperature and other parameters during vertical migration might indeed traduce into physiological costs compared to non-migrating conditions (Stich & Lampert, 1984; Svetlichny et al., 2000). In addition, vertical displacements of diel migrators require an additional energetic investment for swimming activity (Alcaraz et al., 2007), although such energy requirements seem to be highly variable among zooplankton, and could represent from negligible to relatively high costs (Torres & Childress, 1983; Morris et al., 1985; Dawidowicz & Loose, 1992). Hence, the assessment of metabolic disadvantages of vertical migration in zooplankton goes beyond the study of the effects of diel temperature variations and will require further exploration in order to determine the overall energetic balance derived from this migratory behaviour.

The absence of physiological costs in *C. typicus* upon short-term temperature fluctuations is especially relevant within a climate change context, as an increased thermal variability in future climates is foreseen (Easterling et al., 2000; Wang & Dillon, 2014). In fact, it is argued that enhanced temperature variability may represent a greater threat to species than global warming itself (Vasseur et al., 2014), although there might be certain groups such as zooplankton that could benefit from increasing temperature variations (Drake, 2005). Still, former research that evaluated climate-driven temperature impacts on terrestrial and aquatic organisms mostly relied on the effects of mean temperatures (Thompson et al., 2013), and the influence of temperature fluctuations on their fitness remain understudied despite their critical role in future climates (Dowd et al., 2015).

f) Conclusion

Our study provides novel evidence about how neritic marine copepods may not suffer physiological costs from diel temperature fluctuations involved in vertical migration. In this sense, marine copepods might have adaptive mechanisms to optimize the trade-offs derived from their migratory behaviour, thus diminishing the energetic costs traditionally associated to vertical migration. This potential capability of marine copepods may be useful to confront the negative impacts of the increased thermal variations expected with climate change.

g) *Acknowledgements*

We would like to thank Kaiene Griffell for assisting with the maintenance of phytoplankton cultures. This study was framed within the project ZOOTHERM (CTM2017-84288-R), and is a contribution of the Marine Zooplankton Ecology Group (2017SGR 87). Manuel Olivares is supported financially by a FPU Grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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4.5. Summary of the results

Chapter 4.1. Ontogenetic changes in the feeding functional response of the marine copepod Paracartia grani

The type of feeding functional response of the planktonic copepod *Paracartia grani* feeding on the flagellate *Rhodomonas salina* was a Holling Type III function from the early stages (nauplii) to the adults. The foraging effort (i.e. clearance rate) peaked at intermediate food concentrations, whereas the ingestion rates increased with food concentration following a sigmoid curve until becoming steady above saturating food conditions. The maximum clearance and ingestion rates increased with body mass following power scaling factors of approximately 1 from nauplii to copepodites, and 0.75 from nauplii to adults, respectively. Gender also affected the main parameters of the feeding functional response, with maximum clearance and ingestion rates that were 2-3 times higher in females than in males.

Chapter 4.2. Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms

The wild specimens of *Centropages typicus* and *P. grani* showed higher feeding activity at night, but their diel feeding rhythms were quickly lost through multigenerational laboratory rearing. The exposure to predator exudates (jellyfish *Aurelia aurita* and fish *Dicentrarchus labrax*) to copepods reared 8-11 generations in the laboratory did not trigger back the natural rhythmic feeding of *C. typicus*. Moreover, the diel feeding patterns of *C. typicus* showed changes through development, with no day-night differences in the feeding activity of nauplii, but night-skewed feeding in adults. Gender did not affect the intensity of the feeding rhythms in *C. typicus* adults.

Chapter 4.3. Non-lethal effects of the predator Meganyctiphanes norvegica and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod Centropages typicus

The wild specimens of *C. typicus* showed diel feeding rhythms with higher feeding activity at night, but we could not detect any large effect of the euphausiid predator *M. norvegica* on the diel feeding behaviour of copepods. Other factors such as seasonal photoperiod (i.e. daytime length) and food availability, however, seemed to have an influence on the intensity of copepod feeding rhythms. The largest day-night differences in feeding activity were found in shorter days (autumn *vs.* summer) and lower prey availability (limited *vs.* saturation conditions).

Chapter 4.4. The neritic marine copepod Centropages typicus does not suffer physiological costs from diel temperature fluctuations involved in vertical migration
The adult females of *C. typicus* exposed to the fluctuating temperature conditions mimicking vertical migration (14 °C during the daytime and 19 °C at night) showed a better performance (e.g. higher egg production) than those exposed to constant depth conditions (14 °C); however, no significant differences were detected when compared to constant average and surface conditions (17 and 19 °C, respectively).

5. General discussion

This thesis deepens into the patterns of activity of marine planktonic copepods and the driving factors of such patterns, especially those that affect the daily and diel patterns of feeding. The following discussion is a compilation of the interpretations, the contextualization, and the contributions of the major findings of this thesis based on its objectives and underlying hypotheses.

5.1. Ontogenetic changes in the feeding patterns of marine planktonic copepods.

The literature on the feeding patterns of marine planktonic copepods is already extensive. However, most former research only referred to adult females, whereas the patterns of feeding of early stages and males remain understudied. This fact is unfortunate considering the key ecological role of small copepods as the main link between phyto- and microzooplankton, and higher trophic levels in pelagic communities (Lučić et al. 2003, Turner 2004). This thesis further explored the ontogenetic changes in the feeding patterns of marine planktonic copepods, including the analysis of the feeding functional responses (Chapter 4.1) and diel feeding rhythms (Chapter 4.2). We initially hypothesized that differences through development in the metabolic requirements (Ikeda et al. 2001, Almeda et al. 2011), the morphological and behavioural traits (Van Duren & Videler 1995, Paffenhöfer et al. 1996, Paffenhöfer 1998), and the vulnerability to predation (Buskey 1994) could imply important ontogenetic variations in the daily and diel feeding patterns of marine planktonic copepods.

In Chapter 4.1 we provided a complete analysis of the ontogenetic variations in the feeding functional response of the marine calanoid *Paracartia grani*. All the development stages from early nauplii to adults showed Holling Type III responses (Holling 1966). The low foraging efforts (i.e. clearance rates) observed at low food concentrations may reflect a lower efficiency in catching prey when prey are scarce (Paffenhöfer & Stearns 1988), but also the decrease of copepods' feeding activity when capturing prey is energetically more costly than the energy gained by feeding. This implies that in environments with multiple prey, copepods may switch among prey types and foraging strategies depending on the relative prey abundances to obtain the greatest profit (Jonsson & Tiselius 1990, Kiørboe et al. 1996, Gismervik & Andersen 1997, van Leeuwen et al. 2007). In accordance with these assumptions, Holling Type III responses are typically found in planktonic copepods with feeding modes that require higher energy demands (Kiørboe et al. 2018). The feeding mechanisms of the adults of *P. grani*, although less investigated that in other species, show similarities to those of closely-related *Acartia* species (e.g. *Acartia tonsa*) that actively produce feeding currents when feeding on small-sized prey (Tiselius & Jonsson 1990, Saiz & Kiørboe 1995). In the case of *P. grani* nauplii, they typically conduct hopping movements when foraging and may also produce weak feeding currents to finally capture their prey (Henriksen et al. 2007, Bruno et al. 2012). Therefore, we think that despite the profound changes in morphology and behaviour occurring through copepod development, the maintenance of active feeding modes along ontogeny determined the consistency of Holling Type III responses across different life stages.

As predicted, we also found important ontogenetic variations in the feeding patterns of copepods, particularly those referring to maximum clearance and ingestion rates (F_{max} and I_{max}, Chapter 4.1), half-saturation constants (K_m, Chapter 4.1), and diel rhythms (Chapter 4.2). The F_{max} of *P. grani* increased with body mass from early nauplii to late copepodites with a power scaling factor of ca. 1. This factor is higher than the typical 0.75 power factor found for other feeding-related parameters of copepods, such as the ingestion rates (Saiz & Calbet 2007). The steeper increase with body mass in the case of F_{max} may reflect the profound ontogenetic changes enhancing the foraging efficiency from nauplii to copepodites (e.g. increase in size, development of feeding appendages, increased sensitivity to hydromechanical signals) (Kiørboe et al. 1999, Almeda et al. 2018). In the case of adults, however, the F_{max} did not follow the same pattern showed by nauplii and copepodites, which is likely a consequence of their lower feeding efficiency when feeding on small-sized prey, like the *R. salina* offered in our experiments (Paffenhöfer 1971, Berggreen et al. 1988, Landry & Fagerness 1988, Saiz et al. 2014). Unlike the F_{max}, the I_{max} increased with body mass more closely to the universal 0.75 power law defined for animal physiological processes (West & Brown 2005, Saiz & Calbet 2007, Kiørboe & Hirst 2014). In fact, this pattern was observed along the entire ontogeny from early nauplii to adults of *P. grani*, which suggests that predator : prey size ratio could be less relevant to determine I_{max} than F_{max} in planktonic copepods (Frost 1972, Støttrup & Jensen 1990).

In Chapter 4.2 we found that the diel feeding patterns of *C. typicus* clearly differed between nauplii and adults. Whereas the naupliar stages did not reflect day-night differences in their feeding activity, adults (males and females) fed more intensely at night. This finding is in agreement with our initial hypothesis that the diel feeding behaviour of planktonic copepods could be modulated by factors that are size- or stage-dependent, such as predation vulnerability (Landry 1978, Fancett & Kimmerer 1985, Buskey 1994, Kiørboe et al. 1999, Suchman & Sullivan 2000). In this regard, early stages could use their small size as a refuge from visual predation, whereas larger copepods would need to increase their survival chances during daylight hours through other ways like diel vertical migration and/or feeding rhythms (Bollens & Stearns 1992, Neill 1992, Hays et al. 1994, Ohman & Romagnan 2016). Small stages, moreover, might suffer from a high predation pressure by non-visual predators that are more active at night (e.g. large copepods) (Landry 1978, Boersma et al. 2014). Consequently, nocturnal feeding would be less adaptive for nauplii than for adults. This fact, together with the consideration that nauplii are more susceptible to food intake fluctuations than later stages (Calbet & Alcaraz 1997), may explain the ontogenetic variations in the diel feeding patterns of planktonic copepods.

Finally, it is worth mentioning that certain similarities and differences were found in the feeding patterns of males and females. In Chapters 4.1 and 2.2 the feeding rates of *P. grani* and *C. typicus* were significantly higher in females than in males. However, the intensity of the diel feeding rhythms barely differed between genders (approximately 50% higher feeding activity at night). The lower feeding rates of males could not be only explained by differences in size, and may be attributed to other factors, such as the longer times spent in the search of females (Kiørboe 2007, van Someren Gréve et al. 2017). Still, in the case of some planktonic copepods like *Acartia* and related species, the sexual differences in swimming behaviour might not be that prominent (Kiørboe & Bagøien 2005). More likely, other factors such as the different energy allocation in reproduction might help explain the different feeding activity observed between males and females (Saiz et al. 2017).

We can conclude that stage- and gender-specific differences in the feeding patterns of marine planktonic copepods should be highly considered in future research. From the outcome of this thesis, it is evident that evaluations of the grazing impact of copepod populations, either conducted experimentally or *in situ*, must consider the age structure and sex ratio of copepod populations, and the stage-specific vital rates, in order to provide more reliable estimates.

5.2. Driving factors of diel feeding rhythms in marine planktonic copepods

The Chapters 4.2 and 2.3 focused on identifying factors that are involved in shaping the diel feeding behaviour of marine planktonic copepods. As a first approach, in Chapter 4.2 we tracked the changes in the diel feeding patterns of *C. typicus* from wild specimens through the first laboratory-reared generations to assess the relevance of exogenous components. We hypothesized that if feeding rhythms were primarily environmentally-determined, the intensity of feeding rhythms of wild copepods would be weakened or loss through the first generations reared in the laboratory. Indeed, we found that the diel feeding rhythms of wild copepods, with higher feeding activity at night, were quickly lost through multigenerational laboratory rearing. Despite the previous studies that pointed out to endogenous rhythmicity in the diel feeding patterns of copepods (Stearns 1986, Olsen et al. 2000), our findings suggest that the environmental change from sea to laboratory conditions produced the quick variation in the diel feeding behaviour of copepods (Bollens & Stearns 1992, Calbet et al. 1999, Cieri & Stearns 1999). However, it is remarkable that the first laboratory-reared generation of copepods (F1) showed feeding rhythms that resembled those of the wild specimens. The maintenance of feeding rhythms after one generation in the laboratory suggests that epigenetic factors could be involved in shaping the diel feeding patterns of copepods, as the environmental conditions experienced by the predecessors in the sea could have influenced the phenotypic variants of the offspring in the laboratory (maternal effects) (Uller 2008). Although the non-genetic inheritance of phenotypic traits and epigenetic modifications have been investigated in some zooplankton like daphnids (Agrawal et al. 1999, Robichaud et al. 2012, Walsh et al. 2015, Schield et al. 2016), this is still a largely unexplored, but promising field in planktonic copepods (Guyon et al. 2018).

In all the experiments conducted in this thesis, the wild specimens of *C. typicus* and *P. grani* showed diel rhythmicity in their feeding activity (i.e. higher ingestion rates at night), regardless of the limiting or saturating food conditions. This finding opposes Gauld's hypothesis (Gauld 1953), which postulates that copepod feeding rhythms reflect fluctuations in food availability during vertical migration. Predation risk has been traditionally considered one of the major driving factors of diel vertical migration (Bollens & Frost 1989, 1991, Neill 1990, Frost & Bollens 1992), and it might be also involved in the appearance of feeding rhythms (Bollens & Stearns 1992, Cieri & Stearns 1999). In copepods, the risk of being predated is usually enhanced during daylight hours (Tsuda et al. 1998, Torgersen 2001) and, consequently, our initial hypothesis was that the diel feeding rhythms could emerge from restricting the foraging activity to safer periods (i.e. night). However, all the experimental attempts in this thesis to confirm the role of predation risk in triggering copepod feeding rhythms led to negative results. Firstly, in Chapter 4.2 the exposure to chemical cues of the predators *Aurelia aurita* (jellyfish) and *Dicentrarchus labrax* (fish) did not trigger back the diel feeding rhythms in copepods that had lost their natural rhythmic feeding through multigenerational laboratory rearing. In this respect, the effects of predator chemical cues on zooplankton have proved to be less relevant in marine than in freshwater environments (Lass & Spaak 2003). This does not necessarily imply that predation risk does not have any influence on the feeding behaviour of marine copepods, but that the predator-induced responses of marine copepods might not be chemically-mediated, but follow other predatory signals (Bollens & Frost 1989, Bollens et al. 1994). Still, in Chapter 4.3 we also found that the physical presence of the predator *Meganyctiphanes norvegica* (krill) did not have any large effects on the feeding rhythms of freshly-collected copepods. Thus, it seems that the predator effects on copepod feeding rhythms, if any, could be limited and/or highly predator-specific (Wong 1988, Ohman 1990).

In Chapter 4.3 we found that external factors other than predation threat affected the intensity of feeding rhythms in *C. typicus.* Copepods showed feeding rhythms of higher amplitude in autumn (10 h light) than in summer (16 h of light), suggesting that copepods may lower the intensity of their feeding rhythms when safe periods (i.e. dark periods) are too short for feeding to meet metabolic demands. Also, in our experiments we detected that a lower prey availability enhanced the day-night differences in the feeding activity of copepods. This pattern could be attributed to the higher predation risk associated to foraging when copepods need to swim for longer times and cover larger distances to find and capture prey (Saiz et al. 1992, Uttieri et al. 2013, Kiørboe et al. 2014). This pattern is in accordance with the findings by Hassett & Blades-Eckelbarger (1995), but not with those by Calbet et al. (1999), who reported that the copepod feeding rhythms were maintained or weakened at lower food availability. Hence, the effects of food conditions on copepod feeding rhythms still remain debatable.

As a conclusion, it can be said that the different outcomes of this thesis suggest that the diel feeding behaviour of planktonic copepods are primarily environmentally-determined. Although predation risk is accepted as a major driving factor of diel vertical migration, in this thesis we could not confirm any fundamental role of predation threat in the diel feeding patterns of copepods. We cannot fully discard, however, that the appearance of rhythmic feeding as an antipredator response of copepods could be predator-specific. Moreover, additional environmental factors such as food availability and seasonal photoperiod proved to influence the diel feeding behaviour of planktonic copepods.

5.3. The effects of temperature fluctuations on the patterns of activity of marine planktonic copepods.

There is a large bulk of literature addressing the effects of temperature on the activity patterns of marine planktonic copepods. However, little is known about the effects of temperature fluctuations, even though most planktonic copepods may be daily exposed to temperature variations through diel vertical migration. In Chapter 4.4 we found that the neritic marine copepod *C. typicus* exposed to diel temperature fluctuations of vertical migration (10 h at 14 °C and 14 h at 19 °C) showed rates of egg production and respiration that were significantly higher than at constant depth temperature (14 °C); however, no significant differences were detected when compared to the rates of copepods under average or surface conditions (17 and 19 °C, respectively). Our findings indicate that marine copepods like *C. typicus* that experience moderate temperature fluctuations through vertical migration (i.e. differences of 5 °C) do not necessarily suffer physiological costs derived from their migratory behaviour. In this sense, these animals might have physiological mechanisms to compensate diel temperature fluctuations. According to recent proteomic analyses, migrant copepods experience physiological shifts through vertical migration (Maas et al. 2018). The physiological mechanisms that copepods may have to compensate temperature fluctuations could involve the expression of protein variants, or the modification of the protein environment (Clarke 2003). However, this is still a field that requires further research, as the effects of fluctuating temperature of copepod performance are still poorly documented (Lock & McLaren 1970, Vuorinen 1987, Zhou & Sun 2017). Moreover, the investigation of the adaptive mechanisms of copepods to deal with temperature fluctuations should be of high interest in a climate change context, as an increased thermal variability is predicted in future climate scenarios (Easterling et al. 2000, Wang & Dillon 2014). In this respect, zooplankton that are adapted to diel temperature fluctuations might even benefit from the increasing thermal variations (Drake 2005).

The previous studies that addressed the energetic balance of diel vertical migration in zooplankton mostly focused on *Daphnia* and concluded that the fluctuating conditions of temperature and food during migration were less favourable than non-migrating conditions (Orcutt & Porter 1983, Stich & Lampert 1984, Lampert et al. 1988, Loose & Dawidowicz 1994). Based on our findings in Chapter 4.4, marine planktonic copepods might be capable to cope successfully with diel temperature fluctuations when the temperature range is moderate (5 °C). Regarding fluctuations in food availability, in Chapter 4.2 we found that adults of *C.*

typicus were capable of maintaining their total daily ingestions regardless of their diel feeding behaviour (i.e. feeding rhythms), supporting that planktonic copepods could overcome short-term fluctuations in food intake (Davis & Alatalo 1992, Calbet & Alcaraz 1996). However, other factors involved in diel vertical migration like the energy investment in locomotion (Torres & Childress 1983, Morris et al. 1985, Dawidowicz & Loose 1992), as well as the joint effects of the different environmental factors (Stich & Lampert 1984, Svetlichny et al. 2000, Koussoroplis & Wacker 2016) might be also relevant and should be considered for an overall energetic balance of diel vertical migration in planktonic copepods.

6. Conclusions

1. Marine planktonic copepods may show important stage- and gender-specific differences in their daily and diel patterns of feeding. Therefore, future studies addressing the grazing impact of copepod populations should highly consider the age structure and the sex ratio of the populations to obtain more reliable estimates.

2. The diel feeding rhythms of marine planktonic copepods seem primarily environmentally-determined, and the quick loss of natural rhythmic feeding through multigenerational laboratory rearing may bring concern for the use of cultured copepods in experiments involving their diel feeding behaviour.

3. The role of predation risk in the diel feeding behaviour of marine planktonic copepods remains an open question. However, other external factors such as food availability or photoperiod might be involved in the modulation of the intensity of copepod feeding rhythms.

4. Marine planktonic copepods may not suffer physiological costs from temperature fluctuations through diel vertical migration when the range of temperature variations is moderate (e.g. 5 °C). In this sense, marine copepods might have adaptive mechanisms to optimize the trade-offs derived from their migratory behaviour.

7. General bibliography

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8. Annex (published papers)

The following annex contains the papers published within the frame of this Ph. D. thesis:

· Olivares, M.; Saiz, E.; Calbet, A. (2019). Ontogenetic changes in the feeding functional response of the marine copepod *Paracartia grani*. Marine Ecology Progress Series, 616, pp. 25-35. https://doi.org/10.3354/meps12928

· Olivares, M.; Calbet, A.; Saiz, E. (2020). Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms. Aquatic Ecology, 54, pp. 697-709. https://doi.org/10.1007/s10452-020-09768-8

· Olivares, M.; Tiselius, P.; Calbet, A.; Saiz, E. (2020). Non-lethal effects of the predator *Meganyctiphanes norvegica* and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod *Centropages typicus*. Journal of Plankton Research, 42(6), pp. 742-751. https://doi.org/10.1093/plankt/fbaa051

Vol. 616: 25–35, 2019
https://doi.org/10.3354/meps12928

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published May 9

Ontogenetic changes in the feeding functional response of the marine copepod *Paracartia grani*

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ABSTRACT: The feeding activity of copepods is crucial for the structure and functioning of marine ecosystems. Quantification of feeding rates of different copepod life stages across a range of prey densities (functional response) is essential knowledge for improvement of plankton dynamic models. In this study, we conducted experiments to compare the feeding functional responses of nauplii, copepodites, and adults of the marine copepod *Paracartia grani* (formerly *Acartia grani*) when grazing on the flagellate *Rhodomonas salina*. We found that all copepod stages followed a sigmoid curve in their functional responses (Holling Type III model), indicating a metabolic threshold constraining foraging effort at low prey densities. Maximum clearance rates of nauplii and copepodites increased with body mass with a power scaling factor of 1, but maximum clearance rates of adults did not follow the pattern observed for juvenile stages, likely because of the relatively small prey size used in the experiments. Copepod maximum ingestion rates, however, showed allometric scaling along ontogeny that was closer to the typical 0.75 power law and seemed to not be so dependent on prey size. The insights obtained from our study highlight stage-specific differences in copepod feeding activity, and can help improve our capability to estimate the energy flow through copepods in marine food webs.

KEY WORDS: Zooplankton · *Acartia* · Nauplii · Development · Grazing · Ingestion · Clearance rate · Functional trait

1. INTRODUCTION

Zooplankton constitute the main link between primary producers (phytoplankton) and higher trophic levels in marine food webs (Calbet 2001, Calbet & Saiz 2005). They are a key component of the biological pump and play a major role in nutrient recycling in the photic layer (Steinberg et al. 2000, Alcaraz et al. 2010, Turner 2015). Within marine mesozooplankton, copepods typically account for 35–70% of the biomass and represent more than 90% of the abundance (Longhurst 1985). Consequently, the resource exploitation conducted by copepods is crucial for the structure and functioning of marine ecosystems.

Functional responses of copepods are defined as the relationship between their vital rates (e.g. feeding rates, egg production rates) and prey availability (Solomon 1949, Holling 1966). Because of the major role of copepods in the zooplankton, copepod functional responses have great implications for plankton population dynamics (Runge 1988, Gentleman & Neuheimer 2008, Kiørboe et al. 2018). The quantification of functional responses in both field and laboratory studies enables the development of mathematical models to forecast the response of plankton communities to changing species densities (Morozov 2010, Morozov et al. 2012). These predictive models are intrinsically very sensitive to the parameterization of functional responses (e.g. the shape of the functional response) and for this reason, the proper analysis of copepod functional responses becomes an imperative task to improve our capability to assess potential variations in the future composition of plankton communities and the biogeochemical fluxes that occur through them (Gentleman & Neuheimer 2008, Wollrab & Diehl 2015, Egilmez & Morozov 2016).

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Traditionally, the study of feeding activity patterns in copepods has been limited mostly to adults, particularly females. In this respect, feeding functional responses have been well documented for adults of key marine copepod species using a wide variety of prey types, and the records are much more numerous for females than for males (Støttrup & Jensen 1990, Isari & Saiz 2011, Gonçalves et al. 2014, Saiz et al. 2014). Females have been preferred over males in grazing experiments mainly because of their longer life span, higher abundances in natural populations, and fundamental role in recruitment (Kouwenberg 1993, Kiørboe 2006, Kiørboe et al. 2015). However, evidence of distinct feeding behaviours between the sexes (e.g. lower feeding rates in males) demonstrates that gender differences should be taken into account when analysing feeding activity patterns in marine copepods (Lampitt 1978, Uye & Kayano 1994, Saage et al. 2009, Chen et al. 2010, van Someren Gréve et al. 2017b).

Similar to males, juvenile copepods (i.e. nauplii and copepodites) have been greatly neglected in previous research addressing copepod feeding patterns. Juvenile copepods outnumber adults throughout the year in plankton communities (Webber & Roff 1995, Calbet et al. 2001, Turner 2004). They play a key ecological role as major grazers of phytoplankton and small heterotrophs. Therefore, studying their feeding activity is fundamental to evaluating the grazing pressure on the smaller plankton fraction in marine ecosystems (Lučić et al. 2003, Turner 2004, Almeda et al. 2011). The first investigations that focused attention on the feeding patterns of the early stages of copepods focused on their rates of grazing on phytoplankton (Mullin & Brooks 1967, Paffenhöfer 1971, Fernández 1979). In response to increasing interest in this topic, further investigations were carried out on other aspects, such as their foraging behaviour, prey size spectrum, and prey selection (Berggreen et al. 1988, Paffenhöfer 1998, Henriksen et al. 2007, Helenius & Saiz 2017). In terms of feeding functional responses, Fernández (1979) was the first author to provide empirical data on naupliar grazing rates of *Calanus pacificus* across a wide range of prey types and food concentrations. Since then, feeding functional responses have been described for juvenile copepod stages of other genera, such as *Acartia/Paracartia* (Henriksen et al. 2007, Ismar et al. 2008, Helenius & Saiz 2017), *Oithona* (Henriksen et al. 2007, Almeda et al. 2010, Saiz et al. 2014), and *Centropages* (López et al. 2007).

Despite this valuable information, we think that the current literature on marine copepods has not completely addressed the changes in feeding functional response through the entire copepod life cycle. For instance, Almeda et al. (2010) analysed feeding functional responses up to early copepodites, and Saiz et al. (2014) worked with nauplii and adults but not with copepodites. Berggreen et al. (1988) reported changes in the prey size spectrum of *Acartia tonsa* from nauplii to adults, but in their study, the feeding functional responses were not analysed. Thus, it appears that we still lack a complete record regarding how feeding functional patterns change through ontogeny in marine copepods. To the best of our knowledge, only Santer & van den Bosch (1994) have studied this issue, with the freshwater species *Cyclops vicinus*.

Thus, the main goal of this study was to assess ontogenetic changes in the feeding functional response of marine copepods. To accomplish our objective, we conducted grazing experiments in the laboratory with different development stages of the calanoid copepod *Paracartia grani* (formerly *Acartia grani*) and a broad range of prey concentrations of the flagellate *Rhodomonas salina*. *P. grani* is a common neritic species that has been observed from high to middle latitudes in the eastern Atlantic and across the Mediterranean Sea (Boyer et al. 2012). In our study, we compared feeding functional patterns among different *P. grani* life stages (nauplii, copepodites, adults) and sexes (males and females). We expected that the values of the fundamental parameters defining the feeding functional response (maximum clearance and ingestion rates, half-saturation constants) would be influenced by changes along ontogeny in the allometric scaling of metabolic requirements (Ikeda et al. 2001, Saiz & Calbet 2007) and variations in morphological and behavioural traits (Paffenhöfer 1998).

2. MATERIALS AND METHODS

2.1. Experimental cultures

For the experiments, the cryptophyte *Rhodomonas salina* was used as prey (equivalent spherical diameter [ESD]: 6.9 µm, carbon [C] content: 38 pg cell^{-1}, nitrogen [N] content: 7.2 pg cell^{-1}; Helenius & Saiz 2017). A culture of *R. salina* was grown in f/2 medium (Guillard 1983) and kept in the exponential growth phase by diluting 1/3 of the culture daily. For the copepods, a cohort of *Paracartia grani* was obtained from a stock culture maintained at the Instituto de Ciencias del Mar (ICM, CSIC) for more than 10 yr

(Saiz et al. 2015). To produce the cohort, eggs were siphoned out from the bottom of the copepod stock culture and inoculated into 20 l polycarbonate tanks filled with 0.1 µm filtered seawater. Approximately 24 h after the egg addition, unhatched eggs were removed from the bottom with a siphon to ensure the uniformity of the cohort. The abundance of copepods in the cohort tank started with ca. 15 000 nauplii, and decreased as copepods were taken out for experiments (ca. 1000 adults by the end). The cohort was fed ad libitum at concentrations ranging from 7 (nauplii) to 20 (adults) ppm of *R. salina*. *R. salina* is a nutritionally sufficient prey that has been commonly used in the maintenance of *Acartia* cultures (Støttrup & Jensen 1990, Broglio et al. 2003, Saiz et al. 2015). Both the phytoplankton and the copepod cultures used for the experiments were kept in the laboratory in a cold room at 19 ± 1°C and under a 10 h light:14 h dark photoperiod.

2.2. Functional response experiments

In total, 6 feeding functional response experiments were carried out successively using different copepod life stages from the same *P. grani* cohort, encompassing 2 experiments conducted with nauplii, 2 experiments with copepodites, and 2 experiments with adults (one with males and one with females). Prior to each experiment, copepods were collected from the cohort using a 60 µm (nauplii and copepodites) or a 200 µm (adults) mesh and placed in filtered seawater for 2.5–3 h while setting up the experiment. For each functional response experiment, 6 prey concentrations of exponentially growing *R. salina* were prepared. The range of prey concentrations was based on previous knowledge (Calbet & Alcaraz 1997, Helenius & Saiz 2017) to cover limiting to satiating food conditions (500–12 000 cells ml^{-1} for NII–III, 500–14 000 cells ml^{-1} for NV–VI, 500–16 000 cells ml^{-1} for CI–II, 1000–20 000 cells ml^{-1} for CIII–IV, and 1000–24 000 cells ml^{-1} for adults). Prey concentrations were measured with a Beckman Coulter Multisizer III particle counter. A total of 8 bottles were filled with each prey concentration: 2 served as initial bottles (only prey), 3 as control bottles (only prey), and 3 as experimental bottles (prey and copepods). Bottle volumes ranged from 72 ml for early nauplii to 620 ml for adults. All bottles were filled following a 3-step procedure (one-third of the bottle was sequentially filled each time) to ensure homogeneity of prey densities among bottles. Inorganic nutrients (5 ml l^{-1} of f/2 solution, i.e. a final concentration equivalent to f/400) were also added to each bottle to compensate for any differential effect of copepod nutrient excretion on algal growth. Once the bottles were filled with the prey suspensions, the copepods were added to the bottles either as aliquots (nauplii and copepodites) or individually with a pipette (adults). For the aliquots, the concentration of nauplii/copepodites in the filtered seawater was estimated on the basis of replicated counts of subsamples drawn with an automatic pipette, and then the aliquot volume of the copepod suspension required to reach the desired copepod concentration was added to each experimental bottle. To compensate for dilution in the experimental bottles, the same volume of filtered seawater was added to the initial and control bottles. Copepod adults were transferred to suspensions of the respective prey concentration to avoid any dilution effects, and then they were individually removed and transferred to the experimental bottles. Given that different copepod stages (sizes) and different prey concentrations were tested, the number of copepods added to each bottle was adjusted based on unpublished previous data to result in a reduction of ca. 30 % of the prey concentration during incubation. Thus, the number of individuals incubated in the bottles ranged from low to high copepod abundance across the different prey concentrations (80–240 NII–III, 40–160 NV–VI, 40–220 CI–II, 25–90 CIII–IV, 10–35 adult males, and 10–30 adult females). All bottles were sealed with plastic film to avoid air bubbles and capped. The initial bottles were sacrificed at the very beginning of the incubation periods to assess actual initial prey concentrations using the Coulter counter. Some extra copepods were preserved in 4 % formaldehyde for measurement of initial copepod size. Control and experimental bottles were mounted on a rotating plankton wheel (0.2 rpm) and incubated for 20–24 h at 19 ± 1°C and under a 10 h light:14 h dark cycle (photosynthetically active radiation [PAR] 15–34 µmol m^{-2} s^{-1}). After the incubations, suspensions from control and experimental bottles were filtered through a submerged sieve with a 60 µm (nauplii and copepodites) or a 200 µm (adults) mesh to separate copepods from prey. The final prey concentrations in each bottle were measured, as they were for the initial bottles, with the Coulter counter. The experimental copepods were checked for activity under a stereomicroscope and then fixed with formaldehyde (4 % final concentration) for quantification, stage determination, and final body size measurements. Prey sizes were calculated as the geometric mean between initial and final prey volumes measured with the par-

Table 1. Parameters of the feeding functional responses of *Paracartia grani* on *Rhodomonas salina*. Maximum clearance rates (F_{max}), maximum ingestion rates (I_{max}), half-saturation constants (K_m), and prey concentrations at which 90 % of the maximum ingestion rates were reached (C_{90}) are shown for the different copepod stages and sizes (N: nauplii; C: copepodites; Ad: adults). Means ± SE are provided

Copepod stage	Copepod size (µm)	F_{max} (ml ind.$^{-1}$ d^{-1})	I_{max} (cells ind.$^{-1}$ d^{-1})	I_{max} (% body C d^{-1})	K_m (cells ml^{-1})	C_{90} (cells ml^{-1})
NII–III	139 ± 2	0.36 ± 0.02	1623 ± 46	213 ± 6.5	2272 ± 138	6815
NV–VI	238 ± 2	1.29 ± 0.06	5623 ± 185	207 ± 6.5	2188 ± 136	6563
CI–II	457 ± 3	6.25 ± 0.34	21649 ± 659	265 ± 7.6	1733 ± 120	5198
CIII–IV	632 ± 2	15.7 ± 0.98	52761 ± 1290	195 ± 5.4	1684 ± 121	5051
Ad males	922 ± 1	8.98 ± 0.46	67772 ± 1858	68 ± 1.9	3773 ± 243	11318
Ad females	1052 ± 3	15.9 ± 0.57	218457 ± 6401	124 ± 3.3	6877 ± 368	20632

ticle counter. Initial and final copepod sizes were determined by photographing 30–40 nauplii or copepodites and 20–25 adults with an inverted microscope and measuring their body (nauplii) or prosome (copepodites, adults) lengths with ImageJ software (Schneider et al. 2012). Copepod sizes were calculated for each prey concentration as the arithmetic mean between their initial and final mean lengths.

2.3. Data analysis

Average prey concentrations and copepod feeding rates (clearance and ingestion rates) were determined according to equations in Frost (1972).

Holling Type III curves were fitted to copepod feeding rates as a function of prey concentration using the software package SigmaPlot v.14.0. Curve fitting was carried out by non-linear regression following the equations in Helenius & Saiz (2017):

$$F = \frac{I_{max}C}{C^2 + {K_m}^2} \qquad (1)$$

and

$$I = \frac{I_{max}C^2}{C^2 + {K_m}^2} \qquad (2)$$

where F is the clearance rate, I is the ingestion rate, I_{max} is the maximum ingestion rate, C is the prey concentration, and K_m is the half-saturation constant. Estimates of I_{max}, K_m and prey concentration at which 90 % of the maximum ingestion rate was reached (C_{90}) were obtained directly from the Holling Type III model. Maximum clearance rates (F_{max}) were calculated as described in Helenius & Saiz (2017):

$$F_{max} = \frac{I_{max}}{2K_m} \qquad (3)$$

Carbon-specific feeding rates were calculated using the conversion factor 0.221 pg C µm^{-3} for *R. salina* (Helenius & Saiz 2017) and the length–weight relationships for *P. grani*: $W = 3.24 \times 10^{-7}\ L^{2.34}$ for nauplii and $W = 1.12 \times 10^{-10}\ L^{3.58}$ for later stages (E. Saiz et al. unpubl. data), where W is the copepod mass in µg C, and L is the body (nauplii) or prosome (copepodites and adults) length in µm.

The relationships between copepod body mass and maximum feeding rates (clearance and ingestion rates) were adjusted to a power function. The power fits were obtained from linear regression after log transformation of the variables using the software KaleidaGraph 4.5.4. To improve the robustness of the fitting, for this analysis, we added additional estimates of I_{max} of *P. grani* obtained from unpublished experiments (M. Olivares et al. unpubl. data) conducted under similar conditions.

3. RESULTS

The average size of the copepods used in the functional response experiments ranged from 139 µm in early nauplii to 1052 µm in adult females (Table 1). Mean prey size was similar in all experiments (range 7.1–7.4 µm ESD). All stages of *Paracartia grani* showed a Holling Type III feeding functional response when grazing on *Rhodomonas salina*. Clearance rates peaked at an intermediate prey concentration and decreased at lower and higher concentrations (Fig. 1). F_{max} increased with stage/size, from 0.36 ml ind.$^{-1}$ d^{-1} for early nauplii to 15.9 ml ind.$^{-1}$ d^{-1} for adult females; the F_{max} values for adult males were, however, lower than the estimates for late copepodites and females (Table 1). Ingestion rates increased with prey concentration following a sigmoid curve and became steady after reaching food saturation (Fig. 1). I_{max} increased from ca. 1600 cells ind.$^{-1}$ d^{-1} (ca. 0.08 µg C ind.$^{-1}$ d^{-1}) in early nauplii to ca. 218 000 cells ind.$^{-1}$ d^{-1} (ca. 9.12 µg C ind.$^{-1}$ d^{-1}) in adult

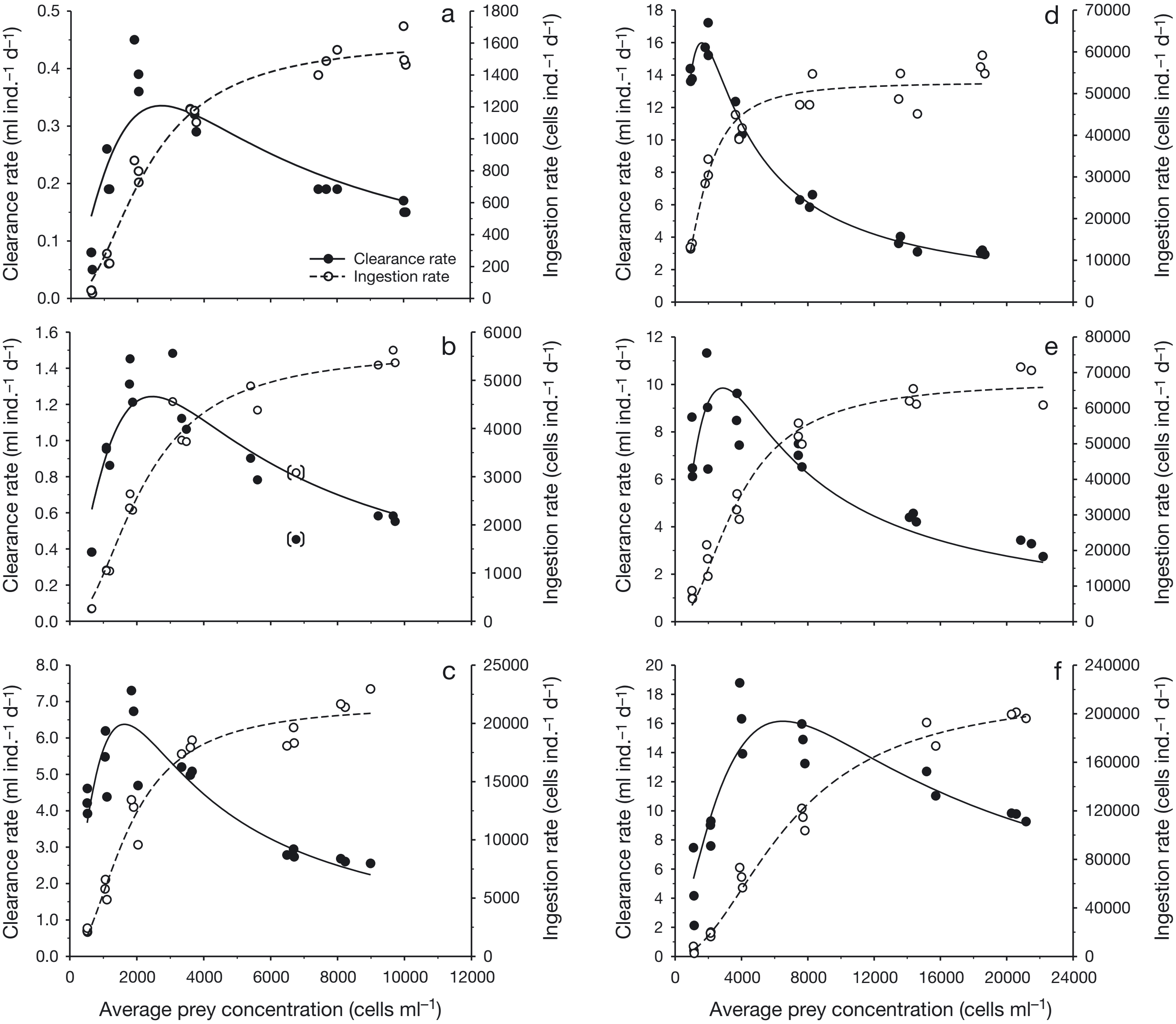

Fig. 1. Functional responses of (a) nauplii II–III, (b) nauplii V–VI, (c) copepodites I–II, (d) copepodites III–IV, (e) adult males, and (f) adult females of *Paracartia grani* feeding on *Rhodomonas salina*. Holling Type III curves were fitted to the clearance and ingestion rates as a function of the average prey concentration. Data in brackets are excluded from the model

females (Table 1). The I_{max} values for females were approximately 3 times higher than those of males.

Fig. 2 shows the power fits between copepod body mass and copepod F_{max} ($r^2 = 0.991$, $p < 0.01$; Fig. 2a) and I_{max} ($r^2 = 0.967$, $p < 0.001$; Fig. 2b). F_{max} of nauplii and copepodites increased with body mass with a scaling factor of 1.09 ± 0.07, whereas the fit between body mass and I_{max} had an exponent of 0.83 ± 0.05 considering all copepod life stages. F_{max} of adults did not follow the general pattern observed for nauplii and copepodites.

In terms of mass-specific rates, the maximum daily ingestion ranged from ca. 210% of the body carbon in nauplii to 265% of the body carbon in early copepodites and then declined to 68 and 124% of the body carbon in adult males and females, respectively (Fig. 3, Table 1). Adults became satiated at much higher prey concentrations than copepodites and nauplii: K_m values were 3773 cells ml^{-1} (ca. 170 µg C l^{-1}) and 6877 cells ml^{-1} (ca. 290 µg C l^{-1}) for males and females, respectively, and less than 2300 cells ml^{-1} (ca. 110 µg C l^{-1}) for juvenile stages (Table 1). The K_m values for copepodites (CI–II and CIII–IV) were lower than those for nauplii. The food concentrations at which 90% of satiation (i.e. the maximum ingestion rate) was attained (C_{90}) followed a similar pattern to

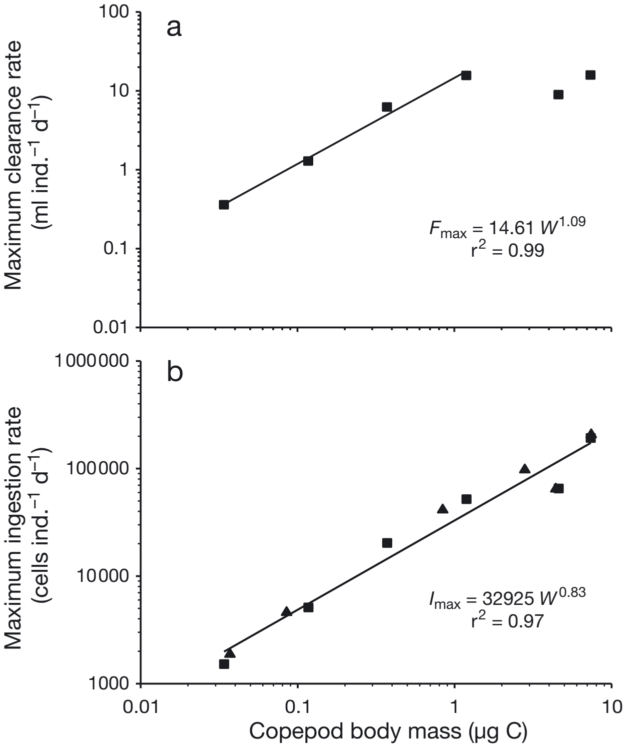

Fig. 2. (a) Maximum clearance rates (F_{max}) and (b) maximum ingestion rates (I_{max}) of *Paracartia grani* feeding on *Rhodomonas salina* with increasing copepod body mass (W). Power function fits and corresponding equations are shown. Note that for the clearance rates, only data on nauplii and copepodites are used for the fit. For the ingestion rates, data from the functional response experiments (■) and additional unpublished trials (▲) are pooled together

that of K_m: decreasing from nauplii to copepodites and substantially increasing in adults (Table 1).

4. DISCUSSION

4.1. Ontogenetic changes in the feeding functional response

We examined changes in the feeding functional response of the marine copepod *Paracartia grani* through ontogeny and focused on relevant ecological aspects, such as the shape and key parameters of the functional response (i.e. I_{max}, F_{max}, K_m). Previous studies have also addressed ontogenetic changes in feeding patterns of marine copepods, but they focused on other aspects of feeding, such as the prey size spectrum (Berggreen et al. 1988), or did not analyse functional responses through the whole developmental range from nauplii to adults (Almeda et al. 2010, Saiz et al. 2014).

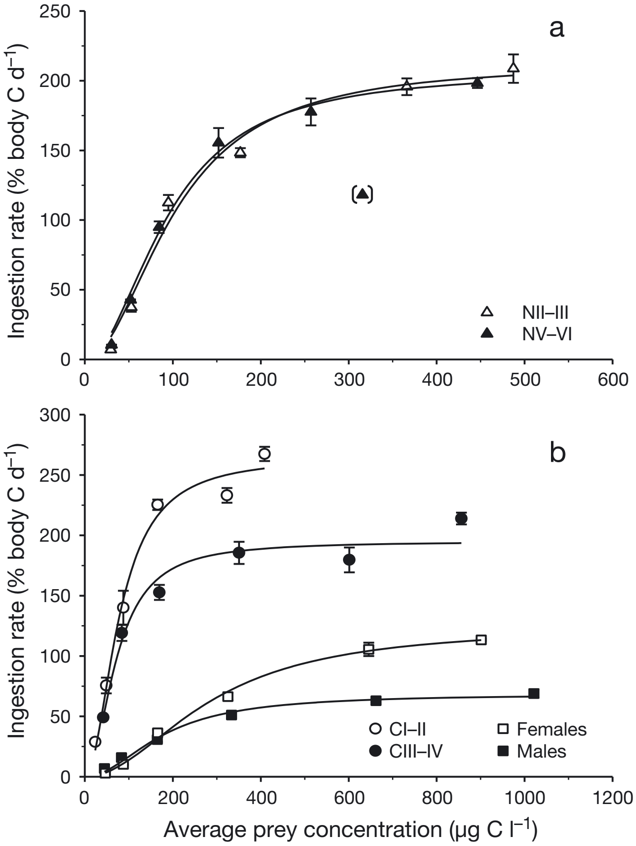

Fig. 3. Carbon-specific functional responses of (a) nauplii and (b) copepodites and adults of *Paracartia grani* feeding on *Rhodomonas salina*. Holling Type III curves were fitted to the carbon-specific ingestion rates as a function of the average prey concentration. Means and SE bars are shown. Data in brackets are excluded from the model

The functional response of an organism shows its capability to exploit environmental resources and the potential flux of energy into secondary production (somatic growth, egg production). We found that the feeding functional response of *P. grani* grazing on *Rhodomonas salina* consistently followed a Holling Type III pattern in all life stages. Holling Type III responses have been traditionally linked to predator–prey systems where predators may control prey densities by switching to alternative prey types according to relative prey abundances, exploiting the more abundant resources and thereby ensuring a basal level of prey diversity in the system (Gismervik & Andersen 1997, Van Leeuwen et al. 2007). However, Holling Type III responses are not restricted to multiple-prey systems, as they have been reported in numerous single-prey experiments (Kiørboe et al. 2018). In our study, we conducted experiments with one algal species and inferred a clear Type III response in all cases. The interpretation of Type III responses in single-prey scenarios is that predators stop actively searching for prey when the energy gained by feeding would not compensate for the energy invested in capturing prey (in our study, this threshold would correspond to approximately 2000 cells ml^{-1} for nauplii and copepodites, and 4000–6000 cells ml^{-1} for adults). Accordingly, this foraging strategy has been proposed to be adaptive for copepods associated with productive environments where prey availability is high, such as coastal environments (Paffenhöfer & Stearns 1988). A recent analysis by Kiørboe et al. (2018) noted that in single-prey environments, Holling Type III responses are more typical of copepods with feeding strategies that involve higher metabolic costs (for instance, creating feeding currents to enhance the encounter rate with prey), while less active swimmers, such as ambush-feeding copepods, generally show a Holling Type II response. Adults of *Acartia* species (sensu lato) normally produce feeding currents when feeding on relatively small prey (Jonsson & Tiselius 1990, Saiz & Kiørboe 1995). The fact that we observed the same feeding functional pattern throughout the whole ontogeny of *P. grani* suggests that active foraging occurs not only in adults but also in the juvenile stages of this marine species. Nauplii of *P. grani* are known to perform continuous hopping movements during foraging and show a Type III response for a wide range of prey types (Henriksen et al. 2007, Helenius & Saiz 2017). There are no detailed reports regarding the underlying mechanisms involved in *P. grani* nauplii feeding, but in the case of the related species *Acartia tonsa*, nauplii are able to detect a prey item remotely and then capture it by using temporary feeding currents generated by the second antennae and mandibles (Paffenhöfer et al. 1996, Bruno et al. 2012).

Although previous studies (Almeda et al. 2010, Saiz et al. 2014) and our results indicate that the feeding functional response follows the same pattern through copepod development, Santer & van den Bosch (1994) reported ontogenetic differences in the freshwater copepod species *Cyclops vicinus*. They found that the naupliar and early copepodite stages of *C. vicinus* exhibited a Holling Type II response, whereas late copepodites and adults responded in more of a rectilinear pattern. However, their observations must be taken with caution because the rectilinear response in late copepodites and adults might be a consequence of not reaching satiating conditions in the experimental food concentrations. Moreover, it is often difficult to accurately distinguish between Type II and Type III responses in data sets, either because very low food concentrations might not be included in the experimental range or because the large variance in counts at low concentrations masks a Type III response.

4.2. Changes in F_{max} through ontogeny

The F_{max} that we estimated for nauplii II-III (0.36 ml $ind.^{-1}$ d^{-1}) was very similar to the value reported by Helenius & Saiz (2017) for the same copepod species, stage, and prey species (0.33 ml $ind.^{-1}$ d^{-1}). These values were also comparable to the F_{max} estimated by Henriksen et al. (2007) for *P. grani* nauplii II-III grazing on larger prey organisms (0.39 and 0.41 ml $ind.^{-1}$ d^{-1} for *Thalassiosira weissflogii* and *Heterocapsa* sp., respectively).

The F_{max} of *P. grani* increased with body mass from nauplii to late copepodites to the power of 1.09, which is in accordance with the scaling factor of 0.93 determined for the clearance rates of the closely related species *A. tonsa* when grazing on *Rhodomonas baltica* (Berggreen et al. 1988) and the value of 1 reported for F_{max} across various zooplankton taxa (Kiørboe 2011, Kiørboe & Hirst 2014). F_{max} describes the capability of a predator to locate and capture prey at low prey densities when factors such as handling time or gut filling are not relevant. Compared to nauplii, copepodites have more developed feeding appendages that can produce larger and stronger feeding currents to capture prey and scan larger volumes of water (Paffenhöfer 1971). Therefore, higher feeding efficiency in copepodites may result in a steeper increase in F_{max} with body mass compared to the typical allometric scaling factor of 0.75 found for other parameters, such as I_{max} (see Section 4.3).

The F_{max} of adults did not follow the general pattern observed in juvenile stages, as the F_{max} of males and females (ca. 9 and 16 ml $ind.^{-1}$ d^{-1}, respectively) were not higher than that of late copepodites (ca. 16 ml $ind.^{-1}$ d^{-1}). We think that this discordance in adult clearance rates is due to the relatively small size of the prey, *R. salina*. It is known that the optimal prey size of marine copepods changes with body size and through ontogeny (Berggreen et al. 1988, Landry & Fagerness 1988, Saiz et al. 2014). It could be argued that the use of the same prey type throughout development may affect the interpretation of our results. In fact, our estimates of the F_{max} for adult females were 4–7 times lower than those previously reported for *P. grani* females feeding on larger prey organisms, such as *T. weissflogii* (ca. 13 µm ESD, 70 ml $female^{-1}$ d^{-1}; Saiz et al. 1992), *Heterocapsa* sp.

(ca. 13 µm ESD, 66 ml female^{-1} d^{-1}; Isari et al. (2013), and *Oxyrrhis marina* (ca. 17 µm ESD, 111 ml ind.$^{-1}$ d^{-1}; Calbet et al. (2007). In Table 1, we can also see that adults showed the highest K_m and C_{90} values through development, indicating that the discrepancy between optimal prey size and predator size was the greatest among all life stages. Therefore, it is likely that if we had used larger prey in the experiments with adults, $F_{\max}$ would have conformed to the power-of-1 scaling pattern throughout development.

4.3. Changes in $I_{\max}$ through ontogeny

Although the $I_{\max}$ that we estimated for nauplii II-III was comparable to the value reported by Helenius & Saiz (2017) for the same copepod species, stage, and prey species (1623 and 1234 cells ind.$^{-1}$ d^{-1}, respectively), our estimate of the carbon-specific $I_{\max}$ (ca. 213% body C d^{-1}) was much higher than the value found in their study (74% body C d^{-1}). This large difference in mass-specific rates relies mostly on the fact that the carbon biomass that we extrapolated for our nauplii (34 ng C ind.$^{-1}$) was substantially lower than the measured biomass in their study (62 ng C ind.$^{-1}$), even though the average naupliar sizes were very similar (139 and 143 µm, respectively). Our estimate, nonetheless, was closer to the maximum daily ingestions reported by Henriksen et al. (2007) for *P. grani* nauplii feeding on *T. weissflogii* and *Heterocapsa* sp. (185 and 299% body C d^{-1}, respectively). In this regard, Henriksen et al. (2007) determined that *P. grani* nauplii, with an average body length of 165 µm, contained 40 ng C animal^{-1}, which indicates that the carbon content values measured by Helenius & Saiz (2017) might have been anomalously high.

The maximum daily ration that we estimated for adult females (124% body C) fell within the range of 103–188% d^{-1} found in the literature for the closely related species *A. tonsa* at same experimental temperature and when provided with the same prey species (Kiørboe et al. 1985, Toudal & Riisgård 1987, Støttrup & Jensen 1990, Thor & Wendt 2010). This value was also close to the specific rates reported for *P. grani* feeding on other prey, such as *Heterocapsa* sp. (ca. 13 µm ESD, 147% d^{-1}; Isari et al. 2013) and *Oxyrrhis marina* (ca. 17 µm ESD, 149% d^{-1}; Calbet et al. 2007). It is worth noting that across prey sizes, maximum mass-specific ingestion rates show a much narrower range of variability in comparison with $F_{\max}$, which can differ by a factor of up to 7 (see Section 4.2). In this regard, Frost (1972) previously reported that the $F_{\max}$ of *Calanus pacificus* varied between 91 and 223 ml ind.$^{-1}$ d^{-1} across a range of prey diameters between 11 and 87 µm, whereas $I_{\max}$ remained fairly constant between 24.2 and 27.1 µg C ind.$^{-1}$ d^{-1}. $F_{\max}$ is very dependent on the predators' perception of prey, which is particularly sensitive to prey size and motility (Saiz et al. 2014, Almeda et al. 2018). In contrast, $I_{\max}$ appears to be not greatly influenced by prey size (Frost 1972) and is more constrained by factors such as copepod gut volume, digestion time, and gut turnover rate (Hassett & Landry 1988, Henriksen et al. 2007). Handling times, as a parameter in Holling equations, are typically also considered for the satiation effect, but in copepods they are generally too short to constrain food intake (Tiselius et al. 2013, Kiørboe et al. 2016). The fact that specific $I_{\max}$ values are not very dependent on prey size helps to explain the exponent of 0.83 in our allometric analysis. This exponent, although slightly higher, was not significantly different from the expected value of 0.75 (*t*-test, $p > 0.05$) according to the 3/4 power law accepted for most organisms, including marine calanoid copepods (West & Brown 2005, Saiz & Calbet 2007, Kiørboe & Hirst 2014). Thus, it appears that the use of a single prey size in our experiments did not result in any substantial bias in the estimation of $I_{\max}$.

In relation to sex differences, mass-specific $I_{\max}$ values for males were ca. 1.8 times lower than those of females (68 and 124% of body C d^{-1}, respectively). Sex-related differences in feeding rates have been previously reported for the same copepod species (Saiz et al. 1992) and other copepod species (Saage et al. 2009, van Someren Gréve et al. 2017b). On the one hand, some authors have attributed the relatively lower feeding rates of males to size differences between the sexes (Bautista et al. 1988, van Someren Gréve et al. 2017b). However, our allometric analysis (Fig. 2) shows that $I_{\max}$ values for *P. grani* males were lower than expected for a copepod of their size. In fact, the feeding rates of late copepodites were also higher than those of males. On the other hand, previous studies state that in some copepod species, adult males are more active swimmers than females and spend a substantial fraction of their time searching for a mate to the detriment of feeding (Kiørboe 2007, Almeda et al. 2017, van Someren Gréve et al. 2017a). We do not know whether sex-related differences in swimming activity could explain the low feeding rates found in *P. grani* males, but it seems unlikely given that the males and females of the related species *A. tonsa* appear to display similar swimming patterns (Bagøien & Kiørboe 2005). We think that the differences in maximum feeding rates between the sexes may not rely on their swimming behaviour but rather on differences in the allocation of energy to the reproductive process. In this regard, *P. grani* females can exhibit high reproductive output under optimal food conditions that would require higher ingestion rates (Saiz et al. 2015, Saiz et al. 2017).

5. CONCLUSIONS

Our study provides empirical data about the ontogenetic changes that marine copepods show in the exploitation of food resources. The insights obtained can help improve modelling capabilities to estimate energy transfer through copepod populations with different stage compositions in pelagic food webs. We have shown that the functional response of the copepod *Paracartia grani* when feeding on *Rhodomonas salina* followed a common pattern (Holling Type III) from early nauplii to adults. F_{max} increased with body mass with a power scaling factor of 1, except for the adult stages, which deviated from that common allometric trend, presumably due to the relatively small prey size. I_{max} scaled to body mass with a factor of 0.8, close to the typical value of 0.75 found in many animals. In this case, prey size did not seem to substantially affect our estimates of I_{max} along ontogeny. We suggest that future studies addressing feeding activity of copepods should also consider the changes in prey size spectrum along ontogeny. In addition, we think that plankton dynamic models would benefit from assessments in multiple resource scenarios, in which selectivity patterns and switching responses can modulate the foraging behaviour of marine copepods.

Acknowledgements. We thank Kaiene Griffell for assisting with culture maintenance. This study was framed within the project FERMI (CGL2014-59227-R, MINECO/AEI/FEDER, UE) and is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). M.O. was supported financially by an FPU grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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Editorial responsibility: Shin-ichi Uye,
Higashi-Hiroshima, Japan

Submitted: July 17, 2018; Accepted: March 7, 2019
Proofs received from author(s): April 4, 2019

Aquat Ecol (2020) 54:697–709
https://doi.org/10.1007/s10452-020-09768-8

Effects of multigenerational rearing, ontogeny and predation threat on copepod feeding rhythms

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Received: 22 November 2019 / Accepted: 1 April 2020 / Published online: 15 April 2020

Abstract Marine planktonic copepods frequently exhibit diel feeding cycles coupled with vertical migrations. However, copepod feeding rhythms can be influenced by factors others than different food availability between layers. In this study, we determined the changes in the diel feeding behaviour of two marine copepod species (*Centropages typicus* and *Paracartia grani*) through multigenerational laboratory rearing, ontogeny, and upon the exposure to predator chemical cues. The wild females of both *C. typicus* and *P. grani* showed marked feeding rhythms with higher ingestion rates at night. The diel rhythms of *C. typicus* were maintained in the first laboratory-reared generation (F1), suggesting maternal effects, but disappeared in the following generations. The *P. grani* females of a long-term culture (> 10 years) also showed no differences in their day–night feeding activity. Ontogenetic variations were detected in the F1 generation of *C. typicus*: feeding rhythms were absent in naupliar stages, but adults fed more intensely at night. In the case of the cultured *P. grani*, in general none of the stages showed feeding rhythms. Laboratory-reared *C. typicus* (8–11 generations) did not recover back the natural cyclic feeding when exposed to jellyfish and fish exudates, indicating that either predation risk does not significantly affect their diel feeding activity or predator-induced responses in marine copepods might not involve chemical signalling. Our study confirms that feeding-related functional traits of marine copepods can experience quick multigenerational changes in the laboratory; consequently, cultured copepods might not be good models for studies involving their diel feeding behaviour.

Keywords Diel rhythms · Functional traits · Marine copepods · Nauplii · Predator exudates

Introduction

Zooplankton are the main link between primary producers and higher trophic levels in pelagic systems (Turner 2004). They play a major role in nutrient recycling within the photic layer (Alcaraz et al. 1994) and contribute significantly to the vertical carbon export to deeper waters, either through sinking faecal pellets and carcasses, or through the active process of diel vertical migration (Longhurst et al. 1990; Longhurst and Harrison 1989; Zhang and Dam 1997). In terms of abundance and biomass, copepods commonly represent a large bulk within migrant mesozooplankton (Longhurst 1985). Migrant copepods typically feed in food-enriched upper layers at night and return

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Handling Editor: Télesphore Sime-Ngando.

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to deeper waters during the daytime (Atkinson et al. 1992; Dagg et al. 1989). Thus, diel vertical migrations are frequently coupled with other activity rhythms (e.g. diel feeding cycles). However, feeding rhythms may also appear in non-migrant copepods and are not strictly linked to upward migrations into food-rich layers (Head et al. 1985; Stearns 1986). Therefore, the ultimate causes for copepod feeding rhythms might differ from those of vertical migrations.

Diel activity patterns of zooplankton are driven by light intensity changes and other factors such as predation risk, food availability, UV radiation, or temperature (Ringelberg 1995; Stearns and Forward 1984; Williamson et al. 2011). The relative importance of these factors to shape zooplankton diel patterns, however, depends on the species and habitats. In particular, levels of exposure and vulnerability to predators seem to highly influence the diel activity patterns of marine copepods. In this regard, Bollens and Frost (1989b) linked the amplitude of vertical migration in *Calanus pacificus* to the abundance of predatory fish. Also, Bollens and Frost (1991) and Bollens and Stearns (1992) found that the diel migrations and feeding rhythms of *Acartia hudsonica* became more pronounced in the presence of fish. Hence, even though prey availability and endogenous rhythmicity have been also proposed as driving factors of copepod diel rhythms (Huntley and Brooks 1982; Stearns 1986), the weakening or absence of diel rhythmicity in the activity of cultured copepods have been ultimately attributed to the lack of potential predators in the environment (Calbet et al. 1999). In this respect, the tracking of multigenerational changes in the diel behaviour of laboratory-reared marine copepods can provide new insights about the exogenous and/or endogenous causes that shape their diel activity patterns.

Copepods have mechanical and chemical sensors in their antennae that can be used to remotely detect predators (Hartline et al. 1996). However, it is not clear whether predator chemicals are really responsible for the predator-induced changes in copepod diel rhythms or, instead, other cues are involved. Cieri and Stearns (1999) and Cohen and Forward (2005b) found that the exposure to predator exudates induced, respectively, a lower daytime feeding in *Acartia tonsa* and *A. hudsonica*, and modifications in the vertical migration patterns of *Calanopia americana*. Contrarily, Bollens and Frost (1989a) and Bollens et al. (1994) reported that migration-related responses of *A. hudsonica* upon predator exposure did not involve chemical signalling. Therefore, the effects of predator chemical cues on copepod diel rhythms still remain debatable. In some cases the absence of responses to predator exudates could be argued with a lack of phenotypic plasticity in copepod populations (Kiørboe et al. 2018). In this respect, copepods reared in the laboratory in the absence of predators for several generations would be a good model to test the long-term phenotypic plasticity of copepod populations to respond to predation threat.

The diel behaviour of copepods can show significant changes through development. In relation to vertical migrations, Osgood and Frost (1994) and Fortier et al. (2001) reported that certain species show diel large-scale movements to deeper layers only during late stages, whereas early stages remain near the surface or are evenly distributed through the water column. Also, Atkinson et al. (1992) and Huntley and Brooks (1982) found that five species of copepods increased the amplitude of their vertical movements from nauplii to adults. Hence, smaller stages with lower swimming capacity might depend more on their reduced size to avoid visual predation, whereas larger stages would need to compensate their increased predation susceptibility during the daytime through downward migrations to deeper, darker layers (Buskey 1994; Neill 1992). It is not known, however, whether changes through development in diel migration patterns of marine copepods are associated to variations in their diel feeding behaviour. Although large marine copepods usually show higher feeding activity at night, it may well be that early development stages, closer in size to protozoan microzooplankton, find more advantageous to follow reverse diel feeding rhythms to avoid predation by adult copepods (Arias et al. 2017; Jakobsen and Strom 2004). Therefore, it can be hypothesized that copepod nauplii might show diel feeding patterns that are different from those of copepodites and adults.

Because of the key ecological role of copepods in aquatic ecosystems, the study of their feeding patterns and factors that modulate their diel behaviour is essential knowledge to improve our capability to determine the energy flux into secondary production in marine and freshwater environments. For that purpose, in this study we analysed the effects of multigenerational laboratory rearing, ontogeny and exposure to predator exudates on the diel feeding behaviour of two marine calanoid copepod species (*Centropages typicus* and *Paracartia grani*). Firstly, we monitored variations in the day and night feeding rates of field-collected (wild) and laboratory-reared descendant copepods. Secondly, we compared the diel feeding patterns of different copepod life stages from the same generation. Finally, we tested whether the presence of predator exudates (from both visual and non-visual predators) could trigger back diel feeding rhythms in copepod populations that had lost their natural rhythmic feeding after multiple-generation rearing.

Materials and methods

Prey cultures

The algae *Rhodomonas salina* and *Heterocapsa* sp., and the heterotrophic dinoflagellate *Oxyrrhis marina* were used as prey for copepod cultures and experiments. The algae were grown in f/2 medium (Guillard 1983) and maintained in the exponential growth phase by diluting 1/3 of the cultures daily. *O. marina* was grown in Guillard's trace metal stock solution (Guillard 1983) and fed with *R. salina*. Cultures of *O. marina* were unfed for two days before the experiments to ensure that *R. salina* had been depleted when the copepod incubations started. All phytoplankton and microzooplankton were maintained in a climate-controlled room at 19 ± 1 °C and 10 h: 14 h light: dark photoperiod (average PAR during the daytime: 90 μmol m^{-2} s^{-1} for algae, 10 μmol m^{-2} s^{-1} for *O. marina*).

Copepod collection and cultures

Wild and laboratory-reared copepods of the species *Centropages typicus* and *Paracartia grani* were used for experiments. Wild specimens (F0 generation) were collected with a Juday-Bogorov plankton net fitted with 200-μm mesh in coastal waters off Barcelona (*C. typicus*, May 2017) and the harbour of El Masnou, a town located 20 km north of Barcelona (*P. grani*, October 2018). In the laboratory, adults of both species were sorted out with a pipette under a stereomicroscope and kept in 15-L polycarbonate tanks with 0.1-μm filtered seawater and food. Copepods were fed ad libitum with either *O. marina* (> 8 ppm, *C. typicus*) or *R. salina* (> 5 ppm, *P. grani*) for 1–2 days before experiments. The temperature and light regimes were those found at sea during sampling: 16 ± 1 °C and 13.5 h: 10.5 h light:dark cycle for *C. typicus*, and 19 ± 1 °C and 10 h: 14 h light:dark cycle for *P. grani*.

For laboratory-reared copepods, up to 11 new generations of *C. typicus* (F1–F11, approx. 1 month per generation) were produced from the eggs spawned by the collected wild specimens (F0) and the following generations (batches of 10,000–20,000 eggs per generation). Eggs were siphoned out from copepod tank bottoms and placed in new tanks with filtered seawater at same temperature and light conditions as the original cultures. These cohorts were fed ad libitum with *R. salina* and *O. marina* during naupliar stages, and with *O. marina* and *Heterocapsa* sp. during copepodite and adult stages. In the case of *P. grani*, the laboratory-reared cohort was obtained from a copepod stock culture kept at our institute for more than 10 years (Fn generation). This cohort was fed with *R. salina* through the entire development.

Experiments to assess the effect of multigenerational rearing on copepod feeding rhythms

The presence and magnitude of diel feeding rhythms (i.e. differences between day and night feeding rates) were analysed in wild (F0) and laboratory-reared (F1, F3, F5, F6, F8, F11) females of *C. typicus*, and wild (F0) and laboratory-reared (Fn) females of *P. grani*. In the case of *C. typicus*, two independent experiments were carried out for each studied generation, except for F5 (only one experiment).

Experiments consisted of paired day and night incubations with copepods and prey. Prior to incubations, copepods were collected from the stock cultures using a 200-μm sieve and transferred to filtered seawater for 1.5 h to ensure complete gut evacuation. Prey suspensions of *O. marina* (experiments with *C. typicus*) or *R. salina* (experiments with *P. grani*) were prepared at satiating or near satiation concentrations (Olivares et al. 2019; van Someren Gréve et al. 2017) using a Beckman Coulter Multisizer III particle counter. Two–three initial bottles (only prey), 3–4 control bottles (only prey) and 3–4 experimental bottles (prey and copepods) were filled with the prey suspensions; then, copepods were transferred into the experimental bottles using a wide-mouth pipette. The number of copepods per bottle was determined to ensure a ca. 20% decrease in the prey concentration by the end of the incubations. The prey and copepod concentrations in the experiments, as well as bottle volumes, can be found in Table 1. All the bottles were sealed with plastic wrap before capped to avoid air bubbles. Control and experimental bottles were mounted on a rotating plankton wheel (0.2 rpm; average PAR 21 µmol m^{-2} s^{-1} in the daytime incubations) and let incubate for 8–9 h in the respective day and night periods. Initial bottles were processed at the beginning of the incubations to determine the actual initial prey concentrations with the Coulter counter. At the end of the incubations, the contents of control and experimental bottles were sieved through a submerged 200-µm mesh to separate copepods from prey. Prey concentrations were then measured with the Coulter counter as for the initial bottles. Copepods were checked for survival and then fixed in 4% formaldehyde solution for counting and size determination. Average prey sizes were calculated as the geometric mean between initial and final volumes registered with the Coulter counter. Preserved copepods were photographed and prosome lengths were measured using the software package ImageJ (Schneider et al. 2012). The average prey and copepod sizes in the experiments are shown in Table 1.

Experiments to assess ontogenetic changes in copepod feeding rhythms

Differences between day and night feeding rates were determined for nauplii II–III, nauplii IV–V and adults (males and females) of the F1 generation of *C. typicus*, and for nauplii II–III, nauplii IV–V, copepodites II–III, copepodites IV–V, and adults (males and females) of the long-term cultured *P. grani* (Fn generation). The experiments with *C. typicus* were carried out twice for each studied stage.

Table 1 Size and concentration of copepods (*Centropages typicus*, *Paracartia grani*) and prey (*Oxyrrhis marina*, *Rhodomonas salina*) in the feeding experiments

Experiment	Copepod species	Generation	Copepod stage	Copepod size (µm)	Prey species	Prey ESD (µm)	Prey conc. (ppm)	Copepod conc. (cop bottle^{-1})	Bottle vol. (mL)
Multiple-generation rearing	*C. typicus*	F0	Females	977 ± 57	*O. marina*	16.8 ± 0.4	9.6 ± 0.4	33 ± 3	620
		F1–F11	Females	1032 ± 33	*O. marina*	17.1 ± 0.5	9.9 ± 0.4	32 ± 3	620
	P. grani	F0	Females	845 ± 2	*R. salina*	7.2 ± 0.02	4.7 ± 0.6	15 ± 1	320
		Fn	Females	1054 ± 2	*R. salina*	7.3 ± 0.03	4.6 ± 0.5	12 ± 3	320
Ontogeny	*C. typicus*	F1	NII–III	166 ± 14	*R. salina*	7.4 ± 0.20	3.6 ± 0.4	281 ± 46	72
		F1	NIV–V	224 ± 18	*R. salina*	7.4 ± 0.19	3.6 ± 0.4	171 ± 39	72
		F1	Males	964 ± 5	*O. marina*	16.7 ± 0.5	9.8 ± 0.4	38 ± 2	620
		F1	Females	1008 ± 7	*O. marina*	16.7 ± 0.5	9.7 ± 0.3	29 ± 2	620
	P. grani	Fn	NII–III	144 ± 2	*R. salina*	7.4 ± 0.10	2.3 ± 0.3	244 ± 44	72
		Fn	NIV–V	207 ± 0	*R. salina*	7.4 ± 0.09	2.3 ± 0.3	97 ± 28	72
		Fn	CII–III	573 ± 31	*R. salina*	7.0 ± 0.02	2.5 ± 0.3	56 ± 9	320
		Fn	CIV–V	803 ± 9	*R. salina*	7.3 ± 0.07	2.9 ± 0.2	57 ± 19	320
		Fn	Males	911 ± 10	*R. salina*	7.3 ± 0.04	4.6 ± 0.5	29 ± 5	320
		Fn	Females	1054 ± 2	*R. salina*	7.3 ± 0.03	4.6 ± 0.5	12 ± 3	320

The volume of the bottles is also provided

ESD equivalent spherical diameter, *N* nauplii, *C* copepodites. Means ± SD are shown

Experiments were conducted following the methodology described in the previous section. Details on prey and predator concentrations and bottle volumes are provided in Table 1. The way of adding copepods into experimental bottles differed depending on stage. Nauplii and copepodites were added as aliquots, and an equivalent volume of filtered seawater was added into the initial and control bottles to correct for any dilution effects. Adults were transferred to prey suspensions and added individually into the experimental bottles using a pipette. In the experiments with nauplii and copepodites, extra copepods were preserved in 4% formaldehyde solution at the beginning of the incubations to determine initial copepod size. At the end of the incubations, the contents of control and experimental bottles were filtered through submerged sieves (37 µm for nauplii, 60 µm for copepodites and 200 µm for adults) to separate copepods from prey. Copepods were preserved, posteriorly photographed, and then total (nauplii) or prosome (copepodites, adults) lengths were measured using ImageJ (Schneider et al. 2012). In the case of nauplii and copepodites, the arithmetic mean between initial and final sizes was computed, whereas only final length was considered for adults. The average sizes of prey and copepod stages in the experiments can be found in Table 1.

Experiments to assess the effect of the exposure to predator exudates on the feeding rhythms of cultured copepods

We carried out additional incubations in the experiments with the F8 and F11 females of *C. typicus* (described previously) to assess the effects of predator exudate exposure. These incubations included a control treatment with filtered seawater, and a treatment with a mixture of filtered seawater (75%) and water with predator exudates (25%). *O. marina* at 10 ppm was used as food source in all the incubations. In total, two experiments with jellyfish exudates (F8 females) and one experiment with fish exudates (F11 females) were conducted.

Water with jellyfish exudates was obtained from a 400-L aquarium containing 24 individuals of the jellyfish *Aurelia aurita* (umbrella diameter 4–6 cm) fed ad libitum with brine shrimps. Water with fish exudates was collected from a 2500-L tank (turnover rate of 2500 L h^{-1}) with 40 individuals of European bass (*Dicentrarchus labrax*, approx. 1200 g per individual) fed three times a week with fodder. The water with predator (jellyfish or fish) exudates was prefiltered through a 20-µm sieve and then vacuum-filtered through a 0.2-µm nucleopore filter. Experimental copepods were acclimatized to one of the water types (i.e. with or without predator exudates) during four days before the experiments. During the acclimation period, 80% of the water was renewed daily in the copepod tanks to ensure that copepods were exposed to fresh predator chemical cues, as the exudates might degrade within 24 h (Dodson 1988; Loose et al. 1993).

Data analysis

Day and night copepod ingestion rates were calculated using the equations from Frost (1972). For carbon-specific feeding rates, the conversion factors 0.179 pg C μm^{-3} and 0.221 pg C μm^{-3} were applied for *O. marina* and *R. salina*, respectively (Helenius and Saiz 2017; Klein Breteler and Gonzalez 1986). To obtain copepod carbon weights, the dry weights of *C. typicus* were estimated using the length–weight relationship established for *Centropages hamatus* by Klein Breteler et al. (1982), and carbon weights were calculated as 40% of dry weights (Champalbert et al. 1973). In the case of *P. grani*, copepod carbon weights were obtained directly from the length–weight relationship reported for this species in Olivares et al. (2019). Total daily ingestions of *C. typicus* females were computed through weighing their day and night ingestion rates by the number of hours in each photoperiod.

A significance level of 0.05 was considered for all the statistical tests. Student's *t* tests were applied to check for significant differences between day and night carbon-specific ingestion rates within generations and life stages. Normality and homoscedasticity assumptions were checked with Shapiro–Wilk and Brown–Forsythe tests, respectively. When datasets did not pass normality or homoscedasticity tests, Mann–Whitney rank sum tests or Welch's *t* tests were applied, respectively. Because of the low number of replicates in the experiments with *C. typicus*, the data from independent experiments of each generation and copepod stage were pooled together for statistical tests. Linear regression was applied to check for any significant change in total daily ingestions of *C.*

typicus females across generations. In the experiments with predator exudates, two-way ANOVA tests were run to assess the effect of the factors day/night and presence/absence of exudates on the ingestion rates of F8 and F11 females.

Results

Changes in copepod feeding rhythms through multigenerational laboratory rearing

The day and night ingestion rates of *C. typicus* females across multiple generations (F0–F11) are shown in Fig. 1a. F0 and F1 females showed, respectively, 63% and 51% significantly higher ingestion rates at night (two-tailed Student's *t* test, $p < 0.01$ for F0; two-tailed Mann–Whitney Rank Sum test, $p < 0.05$ for F1; Fig. 1a). Females of later generations (F3–F11), however, did not show any significant day–night difference in their feeding rates (two-tailed Student's *t* ests, $p > 0.05$ for F3, F6, F8 and F11; two-tailed Mann–Whitney Rank Sum test, $p > 0.05$ for F5; Fig. 1a). Thus, night/day ingestion rate ratios decreased from ca. 1.5 in F0 and F1 to ca. 1.0 in the next generations (Fig. 1b). Total daily ingestions ranged between 115 and 162% body C d^{-1} (average 134% body C d^{-1}) and did not significantly change across generations (linear regression, $p > 0.05$; Fig. 2).

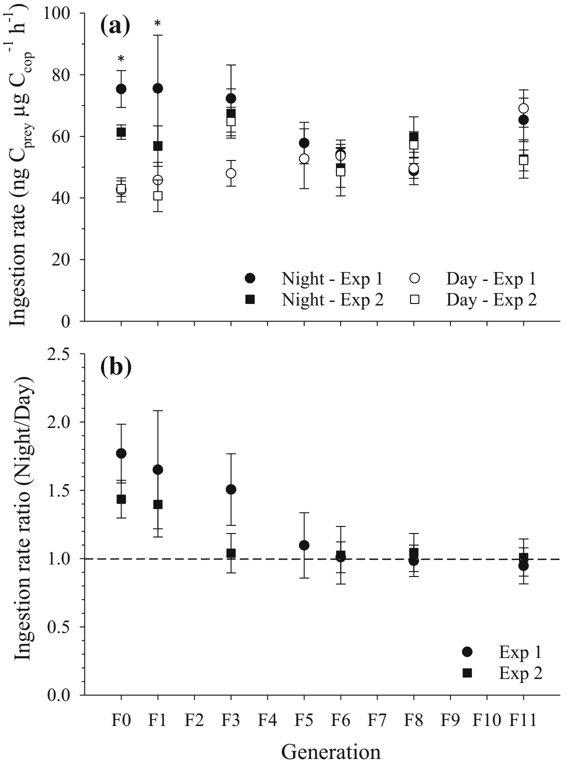

Fig. 1 **a** Day and night ingestion rates, and **b** night/day ingestion rate ratios of wild (F0) and laboratory-reared (F1–F11) females of *Centropages typicus*. Means and SE are shown. Significant differences between day and night ingestion rates are denoted with an asterisk (two-tailed Student's *t* test, $p < 0.01$ for F0; two-tailed Mann–Whitney Rank Sum test, $p < 0.05$ for F1). Dashed line indicates no difference between average night and day ingestion rates (ratio of 1.0)

Regarding the experiments with *P. grani* (F0 and Fn generations), the mean ingestion rates of wild females at night doubled those during the daytime (93.4 and 46.7 ng C_{prey} µg C_{cop}^{-1} h^{-1} for night and day, respectively; two-tailed Student's *t* test, $p < 0.001$; Fig. 3). The ingestion rates of cultured females were 21% higher at night but this day–night difference was not statistically significant (55.5 and 45.8 ng C_{prey} µg C_{cop}^{-1} h^{-1} for night and day, respectively; two-tailed Student's *t* test, $p > 0.05$; Fig. 3).

Ontogenetic changes in copepod feeding rhythms

The diurnal and nocturnal feeding rates of nauplii and adults of *C. typicus* (F1 generation) are shown in Fig. 4a. The mean ingestion rates of NII–III and NIV–V did not show any significant difference between day and night (two-tailed Student's *t* tests, $p > 0.05$; Fig. 4a). However, the mean ingestion rates of adults

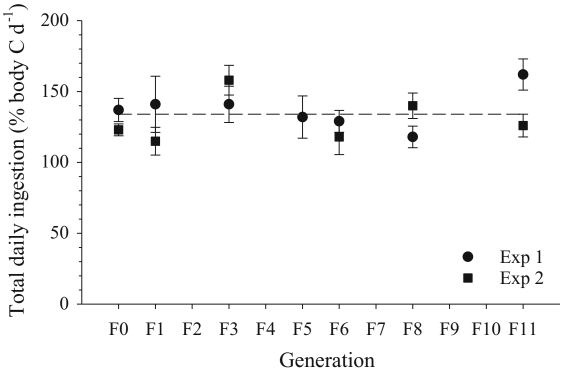

Fig. 2 Total daily ingestions of wild (F0) and laboratory-reared (F1–F11) females of *Centropages typicus*. Means and SE are shown. Dashed line represents the average daily ingestion across generations (134% body C d^{-1})

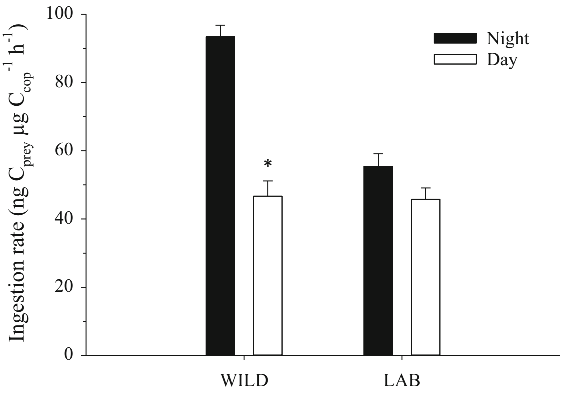

Fig. 3 Day and night ingestion rates of wild ("WILD") and laboratory-reared ("LAB") females of *Paracartia grani*. Means and SE are shown. Significant differences between day and night ingestion rates are marked with an asterisk (two-tailed Student's *t* test, $p < 0.001$). Notice that average copepod sizes were 845 µm and 1054 µm for wild and cultured specimens, respectively

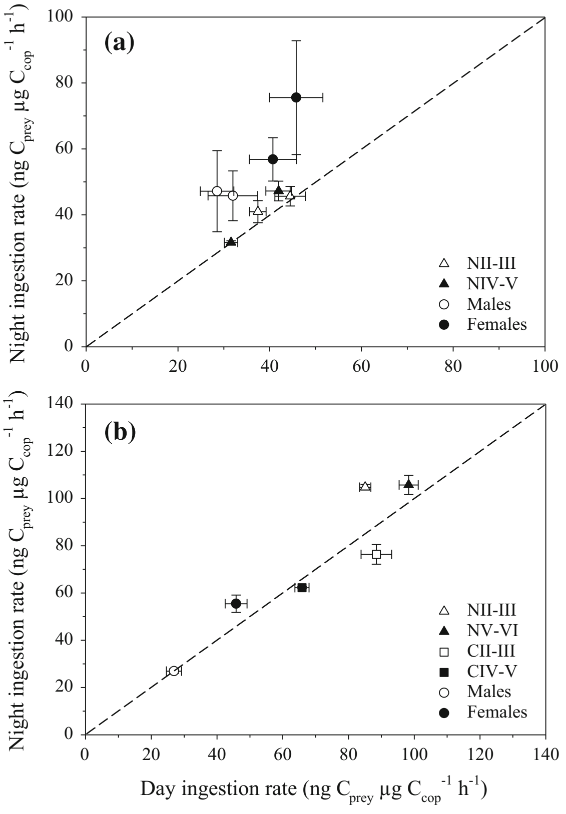

Fig. 4 Day and night ingestion rates of **a** nauplii (N) and adults of *Centropages typicus* and **b** nauplii (N), copepodites (C), and adults of *Paracartia grani*. Means and SE are shown. Dashed line indicates no difference between average day and night ingestion rates

were about 50% significantly higher at night for both sexes (two-tailed Mann–Whitney rank sum test, $p < 0.05$ for females; one-tailed Welch's test, $p < 0.05$ for males; Fig. 4a).

In the experiments with the long-term cultured *P. grani* (Fn generation), in general, none of the stages showed any significant difference between their day and night feeding rates (two-tailed Student's *t* tests, $p > 0.05$ for CII–III, CIV–V, males and females; two-tailed Mann–Whitney Rank Sum test, $p > 0.05$ for NIV–V; Fig. 4b). The only exception was found in nauplii II–III, which exhibited 23% significantly higher ingestion rates at night (day: 85 ± 1.7 ng $\mathrm{C_{prey}}$ µg $\mathrm{C_{cop}^{-1}}$ $\mathrm{h^{-1}}$, night: 105 ± 1.2 ng $\mathrm{C_{prey}}$ µg $\mathrm{C_{cop}^{-1}}$ $\mathrm{h^{-1}}$; Student's *t* test, $p < 0.001$; Fig. 4b).

Effect of exposure to predator exudates on the diel feeding rhythms of cultured copepods

The mean ingestion rates of F8 and F11 females of *C. typicus* exposed to predator exudates ranged between 49.8 and 69.1 ng $\mathrm{C_{prey}}$ µg $\mathrm{C_{cop}^{-1}}$ $\mathrm{h^{-1}}$ and did not show any significant difference to those of the non-exposed females (two-way ANOVA, $p > 0.05$; Fig. 5).

Discussion

Loss of copepod feeding rhythms through multigenerational rearing

In our experiments the natural feeding rhythms of the marine copepods *C. typicus* and *P. grani* were lost through multigenerational laboratory rearing. In the case of *C. typicus*, the absence of rhythms was manifest after only 2–3 generations. Strong positive selection can lead to significant genotypic (and phenotypic) shifts in copepod populations, especially upon exposure to (or lack of) environmental stressors (Jiang et al. 2011; Sun et al. 2014). In our study, the quick loss of diel rhythms could reflect a sharp selection of phenotypes that show constant and continuous feeding when food availability is high. As evidenced in experiments with intermittent food supply (analogous to feeding cycles, i.e. fluctuations in food intake), rhythmic feeding might imply certain fitness costs (Calbet and Alcaraz 1996; Nival et al. 1990). Still, there are copepods that can counteract the negative effects of non-continuous feeding through

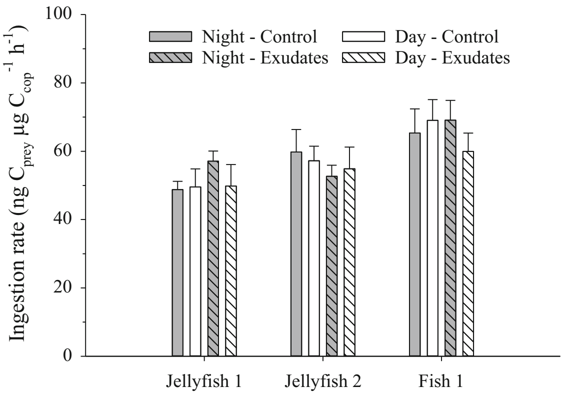

Fig. 5 Day and night ingestion rates of laboratory-reared females of *Centropages typicus* incubated without predator exudates ("control") and with predator exudates ("exudates"). Two experiments with jellyfish exudates and one experiment with fish exudates are shown. Means and SE are provided

enhancing food intake after starvation periods (Kremer and Kremer 1988; Runge 1980). In this regard, Davis and Alatalo (1992) reported that *C. typicus* is indeed capable to cope with short-term food fluctuations (0.5–1 days) in terms of survival, growth, development, and egg production rates. In agreement with this hypothesis, we found that total daily ingestions of *C. typicus* females remained invariable across generations regardless diel feeding behaviour. Thus, it seems unlikely that a strong phenotype selection could have caused the phenotypic shift in our study, as the potential adaptive value of non-rhythmic feeding (i.e. higher daily consumption) was not inferred.

Among the factors that can affect diel activity rhythms in copepods, predation threat has acquired the highest acknowledgement, especially regarding vertical migrations (Bollens and Frost 1989b; Frost and Bollens 1992). In relation to feeding patterns, Bollens and Stearns (1992) found that the amplitude of the diel feeding rhythms of *Acartia hudsonica* greatly increased in the presence of zooplanktivorous fish. Thus, the loss of feeding rhythms through multigenerational rearing could be attributed to the absence of predators in the environment. However, if copepod feeding rhythms were exclusively dependent on predator exposure, we would not expect to find any day–night difference in the food intake of copepods reared in complete predator-free conditions. Yet, we found that the first generation of laboratory-reared females (F1) still exhibited a marked diel feeding cycle despite of being never exposed to predators. Therefore, other factors beyond direct exposure to predators seem to be involved in the modulation of copepod diel rhythms.

Light conditions, prey availability or endogenous rhythmicity could also influence the diel feeding behaviour of marine copepods. About the former, we tried to simulate the light conditions that copepods experience in nature (not only photoperiod, but also light intensity). Thus, the average light intensity in the daytime incubations (ca. 20 µmol m^{-2} s^{-1}) was in the range of the typical values found at the depth where copepods remain during the daytime in the NW Mediterranean (Alcaraz 1988; Estrada 1985). Therefore, we do not think that the experimental light conditions could have caused any significant shift in the diel feeding rhythms of copepods. Regarding food conditions, copepods may show feeding rhythms even at low prey concentrations (Durbin et al. 1990). Still, higher prey availability normally translates into larger amplitudes of both vertical migrations and feeding cycles (Calbet et al. 1999; Huntley and Brooks 1982). In this regard, we kept copepods well-fed through development and experiments, so the magnitude of feeding rhythms should have been kept maximized. We do not know, however, whether the lack of rhythmicity in food availability (as it usually occurs in nature) could have contributed anyhow to the loss of natural rhythmic feeding in copepods. Finally, it is also worth noticing that circadian clocks have been reported to drive the expression of activity rhythms in marine copepods, particularly in vertical migrations (Cohen and Forward 2005a; Häfker et al. 2017) and feeding cycles (Olsen et al. 2000; Stearns 1986). However, the quick disappearance of diel feeding rhythms in our experiments suggests that exogenous cues may play a more important role in this phenotypic response.

The maintenance of diel feeding rhythms in the first reared generation (F1) of *C. typicus* suggests the non-genetic transmission of certain phenotypes through epigenetic mechanisms (Uller 2008). Previous studies with zooplankton demonstrated that exposure to non-lethal chemical cues (e.g. predator or conspecific cues) can lead to transgenerational changes in the functional traits of non-exposed offspring, such as helmet formation or earlier maturation in *Daphnia* (Agrawal et al. 1999; Walsh et al. 2015). Epigenetic mechanisms that mediate non-genetic inheritance have been already investigated in daphnids (Robichaud et al. 2012; Schield et al. 2016), but remain largely unexplored in other zooplankton groups. A recent study by Guyon et al. (2018) reported epigenetic responses (DNA methylation) of copepods to toxic exposure. It seems plausible, therefore, that the presence of diel feeding rhythms in the F1 generation of *C. typicus* could reflect epigenetic modifications determined by the external context experienced by predecessors (F0) in natural conditions (maternal effects).

Lack of response to predator chemical cues

We found that the exposure to predator exudates did not induce the recovery of diel feeding rhythms in laboratory-reared copepods (F8–F11 generations). If predation threat was pivotal to trigger rhythmic feeding in marine copepods, multigenerational rearing could have led to lower intrapopulation plasticity in the response to predatory signals (Cousyn et al. 2001). The loss of capacity to react to predator chemical cues would bring ecologically relevant consequences for species interactions and, at the end, community dynamics (Miner et al. 2005). However, it could happen that predator-induced responses of marine copepods might not involve chemical signalling.

There is a large bulk of research about the significant effects of kairomones on freshwater zooplankton (Lass and Spaak 2003). However, with very few exceptions (e.g. Cieri and Stearns (1999), Cohen and Forward (2005b)), the attempts to use predator chemical cues to induce behavioural changes in marine copepods have failed (Bollens et al. 1994; Kiørboe et al. 2018). This suggests that, unlike in freshwater zooplankton, responses of marine zooplankton to predation threat might be mediated by non-chemical cues (Kiørboe et al. 2018; Ringelberg 1995), whereas chemoreception would be more decisive for other purposes such as food selection or mate finding (Bagøien and Kiørboe 2005; Buskey et al. 2011; Tiselius et al. 2013). We cannot discard, however, that copepod feeding rhythms might not be that influenced by predation threat, and/or that the predators selected for our experiments (jellyfish *Aurelia aurita* and fish *Dicentrarchus labrax*) were unsuitable for the goal of our study. Thus, a non-visual predator like *A. aurita* might not alter copepod diel cycles that might be essentially triggered by visually guided predators (Bollens and Stearns 1992). Regarding *D. labrax*, the diet of adults certainly shows a lower preference over copepods compared to that of juveniles (Rogdakis et al. 2010). In fact, non-lethal effects of predation in copepods and other zooplankters are highly predator-specific (Lüning 1992; Ohman 1990) and may affect behaviour in very different ways (Saiz et al. 1993).

Ontogenetic changes in copepod feeding rhythms

Diel feeding rhythms were absent in naupliar stages but appeared in adults of the F1 generation of *C. typicus*. These ontogenetic variations agreed with diel migration patterns, as nauplii either do not migrate or conduct weaker vertical migrations compared to late life stages (Atkinson et al. 1992; Fortier et al. 2001; Huntley and Brooks 1982). The higher predation susceptibility of young stages to non-visual predators (Titelman 2001), more active at night, makes nocturnal feeding much less adaptive to nauplii than to copepodites and adults (Boersma et al. 2014; Ohman 1990; Uye and Liang 1998). Moreover, the lower tolerance of early stages to fluctuations in food intake (Calbet and Alcaraz 1997) indicates that the appearance of feeding rhythms in marine copepods might be dependent on stage-specific metabolic requirements.

Regarding the long-term cultured *P. grani* (> 10 years in the laboratory), in general none of the stages showed any difference in their day–night feeding activity. Contrarily, wild adults of this species typically show higher feeding rates at night (this study; Bautista et al. (1988); Calbet et al. (1999)). As demonstrated in our experiments, laboratory-reared copepods might not conserve their natural rhythmic feeding behaviour after multigenerational rearing. In the case of nauplii II–III, we found an unexpected night-skewed feeding, although the amplitude of such a rhythm was low (23% higher ingestion rates at night). We analysed closely the initial and final stage compositions in that experiment (data not shown in the paper) and found that the stage proportions were really similar at the beginning of the day and night incubations (ca. 90% NII and 10% NIII). However, the final stage composition differed slightly between incubations (50:50 and 40:60 NII:III proportions during the daytime and night incubations, respectively). One could speculate that NII ingestion rates could have been comparatively lower if any yolk remains were still available. Therefore, the day–night differences found in that experiment could be attributed to the larger proportion of NIII at the end of the night incubation.

Conclusions

Our study confirms that feeding-related functional traits of marine copepods can show rapid multigenerational changes in the laboratory. The quick loss of natural rhythmic feeding through rearing suggests that marine copepods might express feeding rhythms upon exogenous cues, and not for endogenous causes, which may bring concern for the use of cultured copepods in experiments involving their diel feeding behaviour. The maintenance of diel rhythms in the first laboratory-reared generation of *C. typicus* indicates epigenetic mechanisms that mediate the non-genetic transmission of phenotypic traits (maternal effects). The evidence of ontogenetic variations in diel feeding behaviour stands out the importance of age-related factors, such as size-dependent vulnerability to predation, for the appearance of diel rhythms in copepods. Finally, the lack of response to the exposure of predator exudates indicates that either predator-induced responses of marine copepods might not involve chemical signalling, or factors other than predation risk can act as driving factors of copepod feeding rhythms.

Acknowledgements We would like to thank Kaiene Griffell for assisting with field sampling and culture maintenance. We also want to thank Ainara Ballesteros and Mercedes Blázquez for allowing the collection of water with jellyfish and fish exudates. This study was framed within the Project FERMI (CGL2014-59227-R, MINECO/AEI/FEDER, UE), and is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). Manuel Olivares is supported financially by a FPU Grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

Author contributions All the listed authors (MO, AC, ES) have contributed to the experimental design, the field and laboratory work, the data analysis/interpretation, and the manuscript preparation. All the listed authors have also approved the final version of this manuscript.

Compliance with ethical standards

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

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Plankton Research
academic.oup.com/plankt

J. Plankton Res. (2020) 42(6): 742–751. First published online November 3, 2020 doi:10.1093/plankt/fbaa051

ORIGINAL ARTICLE

Non-lethal effects of the predator *Meganyctiphanes norvegica* and influence of seasonal photoperiod and food availability on the diel feeding behaviour of the copepod *Centropages typicus*

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Received May 7, 2020; revised October 2, 2020; accepted October 6, 2020

Corresponding editor: Xabier Irigoien

Predators can induce changes in the diel activity patterns of marine copepods. Besides vertical migration, diel feeding rhythms have been suggested as an antipredator phenotypic response. We conducted experiments to assess the non-lethal direct effects of the predator *Meganyctiphanes norvegica* (northern krill) on the diel feeding patterns of the calanoid copepod *Centropages typicus*. We also analysed the influence of seasonal photoperiod and prey availability on the intensity of copepod feeding rhythms. We did not detect any large effect of krill presence on the diel feeding behaviour of copepods, either in day-night differences or total daily ingestions. Seasonal photoperiod and prey availability, however, significantly affected the magnitude of copepod feeding cycles, with larger diel differences in shorter days and at lower prey concentrations. Therefore, the role of non-lethal direct effects of predators on the diel feeding activity of marine copepods remain debatable and might not be as relevant as in freshwater zooplankton.

KEYWORDS: diel rhythms; predator–prey interactions; zooplankton; krill; faecal pellets

INTRODUCTION

Predation threat can trigger a wide variety of responses in animals, such as changes in morphology, physiology and/or behaviour (Lima, 1998; Agrawal, 2001). These predator-induced phenotypic changes have evolved to increase the animal's survival chances, but also bring certain fitness costs in terms of energy acquisition and resource allocation (Sih, 1980; Lima and Dill, 1990; Preisser *et al.*, 2005). Thus, predators can have negative impacts on prey populations not only through direct predation (consumptive effects), but also through the so-called non-consumptive (or non-lethal) effects. Non-lethal effects of predation can even represent a higher cost for prey demography than predation itself (Preisser *et al.*, 2005), and certainly have important ecological implications regarding community dynamics (Werner and Peacor, 2003; Schmitz *et al.*, 2004).

Within marine communities, copepods are a vital link between primary producers and fish (Runge, 1988) and typically account for the highest abundance and biomass among mesozooplankton (Longhurst, 1985). Therefore, non-lethal effects of predators on copepod populations can translate into important cascading effects in marine food webs (van Someren Gréve *et al.*, 2019). Copepod responses to increasing predation risk include changes in their foraging time, swimming speed and reproductive output (Saiz *et al.*, 1993; van Duren and Videler, 1996; Lasley-Rasher and Yen, 2012; Heuschele *et al.*, 2014). Of particular relevance is how predation risk can alter copepod diel behaviour. For instance, predation threat appears to be the major driver of diel vertical migrations in marine copepods (Frost, 1988; Ohman, 1990; Bollens and Frost, 1991; Hylander and Hansson, 2013). Migrant copepods typically stay in food-enriched upper waters at night, and move to deeper, darker layers during the daytime to avoid visual predation.

Besides vertical migration, copepods frequently show other diel activity rhythms involving their feeding, spawning and moulting patterns (Ohman, 1988). About the former, copepods generally show higher feeding activity at night (Atkinson *et al.*, 1992; Dagg *et al.*, 1998). Nocturnal feeding is usually coupled to vertical migrations, but this feeding behaviour is not necessarily a consequence of staying in food-enriched upper layers at night. In fact, rhythmic feeding may also appear in non-migratory copepods (Hayward, 1980; Head *et al.*, 1985). Therefore, diel feeding rhythms of marine copepods might confer an adaptive advantage that is independent of vertical migration.

Feeding in copepods implies higher motility and conspicuousness, which increases their detectability and predation vulnerability (Tsuda *et al.*, 1998; Uttieri *et al.*, 2013; Kiørboe *et al.*, 2014). This especially applies to daylight hours when copepods are more susceptible to visual predation (Tsuda *et al.*, 1998; Torgersen, 2001). Thus, feeding rhythms (i.e. lower daytime activity) have been traditionally considered an antipredator strategy in copepods (Ohman, 1988). Bollens and Stearns (1992) and Cieri and Stearns (1999) found that the planktonic copepods *Acartia tonsa* and *Acartia hudsonica* showed a lower daytime gut fullness when exposed to fish or fish exudates. However, other studies have not found any effect of predation threat on the feeding behaviour of marine copepods (Kiørboe *et al.*, 2018; Olivares *et al.*, 2020). Hence, the predator effects on copepod feeding rhythms still remain unclear. Most former research on this topic relied on predator exudates as predatory signals, even though marine copepods are known to respond to hydromechanical cues generated by predators (Kiørboe *et al.*, 1999; Hwang and Strickler, 2001; Buskey *et al.*, 2011). In this respect, further experiments with copepods exposed to freely swimming predators are necessary to detect predator-induced responses that are not only chemically triggered (e.g. Saiz *et al.* 1993; Tiselius *et al.* 1997).

The intensity of feeding-related antipredator responses of copepods (e.g. feeding rhythms) can depend on other factors besides predation threat. Copepod diel rhythms can show great seasonal variations (Durbin *et al.*, 1995; Irigoien *et al.*, 1998). These seasonal differences could be attributed to changes in the relative length of daylight periods associated to a higher predation threat (Lima and Bednekoff, 1999). Also, copepods can adapt their foraging behaviour to changing food conditions that affect their risk of being predated (Tiselius *et al.*, 1997; Visser, 2007; van Someren Gréve *et al.*, 2019). However, the effect of increasing food availability on copepod feeding rhythms is controversial. For instance, Hassett and Blades-Eckelbarger (1995) found that day-night differences in copepod feeding activity became larger at lower prey concentrations, whereas Calbet *et al.* (1999) reported that lower food concentrations did not affect or led to weaker diel feeding rhythms in some species.

The main goal of our study was to determine the non-lethal effects of predators on the diel feeding behaviour of marine copepods. Additionally, we also analysed the influence of seasonal photoperiod and prey availability on the magnitude of copepod feeding rhythms. We conducted laboratory experiments with the calanoid copepod *Centropages typicus* and the krill *Meganyctiphanes norvegica* as predators. *M. norvegica* acts as a key predator and grazer in pelagic communities of the North Atlantic with a preference for large and medium-sized copepods (Beyer, 1992; Båmstedt and Karlson, 1998; Agersted and Nielsen, 2016), and is a fundamental prey item for larger fish, squids and whales (Schmidt, 2010; Simard and Harvey, 2010; Suca *et al.*, 2018).

METHOD

Experimental organisms

The heterotrophic dinoflagellate *Oxyrrhis marina*, the calanoid copepod *Centropages typicus* and the euphausiid *Meganyctiphanes norvegica* were used for experiments in summer 2018 and autumn 2019.

O. marina was grown in 0.5-μm filtered seawater at 18 ± 0.5°C and fed daily with the cryptophyte *Rhodomonas salina*. *R. salina* was grown in B medium (experiments in 2018, Hansen (1989)) or f/2 medium (experiments in 2019, Guillard (1983)). The cultures of *O. marina* were not fed for 48 h before experiments to ensure the absence of *R. salina* cells during incubations.

Copepods were collected in the Gullmar Fjord (58° 15.7′ N, 11° 26.7′ E, Sweden) using a 250-μm mesh plankton net. In the laboratory adult females of *C. typicus* were isolated using a pipette and kept in 8-L polycarbonate tanks with filtered seawater and food (*O. marina*, > 4 ppm). The sorted copepods were maintained at 14.5 ± 0.5°C under a photoperiod that simulated natural light conditions: 16 h: 8 h light: dark in summer, and 10 h: 14 h light: dark in autumn.

Krill (*M. norvegica*) were collected in the deepest part of the fjord (58° 19.0′ N, 11° 32.6′ E) using an Isaacs-Kidd Midwater Trawl. Upon arrival at the station, the specimens were transferred to a 300-L glass fibre flow-through tank at 10°C and turnover rate 450 L h^{-1}. Krill were kept in constant darkness and fed daily with freshly collected zooplankton from the fjord.

Experimental set-up

Experiments consisted of day and night incubations of copepods (*C. typicus*) feeding on *O. marina* in the absence and the presence of predators (krill *M. norvegica*). Before incubations, copepods were collected from their maintenance tanks using a 200-μm mesh sieve and placed in filtered seawater for ca. 1.5 h to allow gut evacuation. The cell concentration of *O. marina* stock culture was determined with a Z Series Coulter Counter. About 8 to 10 bottles were filled with filtered seawater and *O. marina* was added to each bottle to obtain final prey concentrations of either 5.5–7 ppm (ca. 1900–2700 cells mL^{-1}; high food; five experiments) or 1.0 ppm (ca. 250 cells mL^{-1}; low food; one experiment). The bottle volumes and *O. marina* concentrations in the experiments are shown in Table I. Four of the bottles were used as control bottles (*O. marina* + copepods) and four to six bottles as experimental bottles (*O. marina* + copepods + krill). A total of 30 copepods and one krill were added to each experimental bottle using, respectively, a wide-mouth pipette and an aquarium fish net (except for one experiment, with only 20 copepods per bottle). The copepod densities in the bottles (7.5–13 cop L^{-1}) were comparable to those that can be found in the Gullmar Fjord (Vargas *et al.*, 2002; Tönnesson and Tiselius, 2005). In the case of krill, the experimental densities (0.25–0.4 ind L^{-1}) were higher than the typical average densities of *M. norvegica* in nature (Onsrud and Kaartvedt, 1998; Tarling *et al.*, 1998), but fell within the range of densities reported for dense krill swarms (Nicol, 1986; Kaartvedt *et al.*, 2005). The bottles were then incubated for 8.5–11.5 h in a temperature-controlled room at 14.5 ± 0.5°C and under the seasonal photoperiod specified before (Table I). The bottles were lit from the side to diminish vertical heterogeneity in the distribution of *O. marina* and *C. typicus* due to small-scale migrations during incubations (Alcaraz *et al.*, 2007; Bochdansky *et al.*, 2010; Bollens *et al.*, 2011). After the incubations, the contents of the bottles were sieved through a 200-μm mesh to collect copepods and krill, and then through a 20-μm mesh to collect copepod faecal pellets. The survival of copepods and krill was checked and the number of dead copepods was noted. The bottles with dead krill were discarded for data analysis (2 out of 30 bottles). The length of krill specimens was measured with a ruler. Copepods and faecal pellets were preserved with Lugol's solution for number and size determination. Photos of 20 copepods and 60–70 faecal pellets were taken per treatment (i.e. with and without krill), and the prosome length of copepods and the length and width of faecal pellets were measured with the software ImageJ (Schneider *et al.*, 2012). *O. marina* size was obtained from Coulter Counter data registered at the beginning of the incubations.

The number of replicates was determined based on power calculations and published data on variability. For the predation by krill, Lass *et al.* (2001, Fig. 7C) reported day/night differences in gut fullness determined from the number of copepod mandibles in the gut. The krill contained 39% more mandibles at night and the standard deviation of the gut fullness was ~25% of the mean. To detect a similar difference with a power = 0.8 required 17 replicates from each of day and night (df = 32) in our study. For the faecal pellet production, we used the clearance rates in Calbet *et al.* (1999, Fig. 1) for *C. typicus*, which were 75% higher at night and with a standard deviation ~40% of the mean. With a standard deviation = 40% and a predicted difference of 50% between day and night, a design with 11 replicates from each of day and night (df = 20) was required for a power = 0.8. Since it was not

Table I: Temperature, light conditions, concentrations of prey (Oxyrrhis marina) and copepods (Centropages typicus) and bottle volumes used in the experiments. Mean ± SD are provided

Experiment	Date	Temperature (°C)	Photoperiod (day: night)	Irradiance (µmol photons m^{-2} s^{-1})	Prey conc. (ppm)	Copepods per bottle	Bottle volume (mL)
1	12 Aug	14.7 ± 0.23	16 h: 8 h	2.2 ± 0.21	5.5	20	2 300
2	16 Aug	14.7 ± 0.23	16 h: 8 h	2.2 ± 0.21	7.0	30	2 300
3	24 Aug	14.7 ± 0.23	16 h: 8 h	2.2 ± 0.21	6.0	30	2 300
4	18 Oct	14.4 ± 0.11	10 h:14 h	1.7 ± 0.33	6.5	30	4 000
5	25 Oct	14.4 ± 0.11	10 h:14 h	1.7 ± 0.33	1.0	30	4 000
6	28 Oct	14.4 ± 0.11	10 h:14 h	1.7 ± 0.33	6.5	30	4 000

possible to run all replicates in one experiment, the entire experiments were repeated three times in summer and in autumn.

Pilot experiments—correction factors for data analysis

The krill *M. norvegica* may feed on small-sized phytoplankton and microzooplankton cells (Agersted and Nielsen, 2016), as well as on detritus and sediments (Youngbluth *et al.*, 1989; Lass *et al.*, 2001). Therefore, pilot experiments were conducted to account for any potential effect of krill on *O. marina* concentration and/or copepod faecal pellet accumulation in the incubations.

To check for krill grazing on *O. marina*, 11 bottles of 4 L were filled with acclimatized filtered seawater and adjusted to 1 ppm of *O. marina* following the same methodology as in the main experiments. Among the 11 bottles, three bottles were used as initial bottles (only *O. marina*), four as control bottles (only *O. marina*) and four as experimental bottles (*O. marina* and one krill). The organisms were added to the bottles as described in the previous section. Control and experimental bottles were then incubated for 10 h under the same conditions as in the main experiments, and initial and final *O. marina* concentrations were measured with a Coulter counter.

Two incubations were carried out to determine krill grazing on copepod faecal pellets. Six bottles (first incubation) or 10 bottles (second incubation) of 4 L were filled with acclimatized filtered seawater. Half of the bottles served as control bottles (only faecal pellets) and the other half as experimental bottles (faecal pellets and one krill). Faecal pellets were collected from copepod tanks by siphoning the tank bottoms and removing copepods with a 200-µm mesh. The faecal pellet concentration was estimated by counting three subsamples, and then aliquots containing around 350 faecal pellets were added to each bottle. Krill were transferred to the experimental bottles using an aquarium net. The bottles were then incubated for 10 h in the same conditions as in the main experiments. At the end of the incubations, the krill and the faecal pellets were collected using a 20-µm mesh. The faecal pellets were fixed in acidic Lugol's solution for counting and size determination. A total of 60 faecal pellets per treatment (i.e. with and without krill) were photographed, and length and width measurements were conducted with ImageJ (Schneider *et al.*, 2012).

Data analysis

The feeding activity of copepods was estimated based on their faecal pellet production rates (Nejstgaard *et al.*, 2001; Besiktepe and Dam, 2002). The average pellet volumes were calculated assuming an ellipsoidal shape. Gut evacuation times of copepods (20 min at 14°C, Irigoien (1998)) were subtracted from incubation times because copepod guts were empty before incubations. In the experimental bottles where krill actively predated on copepods, the average number of copepods during incubation was calculated assuming an exponential decrease of copepod abundance following the equations in Frost (1972).

The pilot experiments showed that in 10-h incubations the *O. marina* concentrations in the bottles did not change regardless of the absence or the presence of krill (two-tailed Student's t-tests, $P > 0.05$). However, krill removed 15% of copepod faecal pellets during incubations (randomized block design (RBD) analysis of variance (ANOVA), $F(1,13) = 10.76$, $P < 0.01$). Thus, faecal pellet production rates of copepods were corrected assuming a pellet removal by krill of 1.5% per hour.

After data correction, RBD ANOVA tests with experiment as block factor were conducted to check for significant effects of the factors day/night and absence/presence of predator (krill) on copepod pellet production rates. RBD ANOVAs were applied to each set of experiments with the same photoperiod and prey concentration (i.e. experiments in summer at high food, and experiments in autumn at high food, Table I). For the only experiment at low food availability (1 ppm), a two-way ANOVA was used instead. Additionally, a two-way ANOVA was conducted to check for significant differences between seasonal photoperiod (16:8 h *vs* 10:14 h) and prey availability (high *vs* low) in the magnitude of copepod feeding rhythms (i.e. night/day ratios of pellet production rates). Finally, a two-way ANOVA was applied to krill predation rates to test significant differences between day and night, and between seasons (i.e. photoperiod). All datasets passed normality and homoscedasticity assumptions according to Shapiro–Wilk and Brown-Forsythe tests, respectively.

RESULTS

The krill *Meganyctiphanes norvegica* was actively feeding on copepods in our experiments. The average predation rates ranged 0.1–0.6 cop krill^{-1} h^{-1} and no significant differences were detected between day and night, or between seasons (two-way ANOVA, F(1,54) = 1.51 and $P > 0.05$ for day/night, F(1,54) = 3.61 and $P > 0.05$ for photoperiod; Fig. 1). The interaction between factors was also not significant (two-way ANOVA, F(1,54) = 1.21, $P > 0.05$).

Table II shows the sizes of copepods and prey, as well as the faecal pellet production rates of copepods and the pellet volumes in the incubations. All the krill specimens had a body length of ca. 40 mm. The pellet production rates of copepods were significantly higher at night in all the experiments (summer at high food: RBD ANOVA, F(1,50) = 70.63, $P < 0.001$; autumn at high food: RBD ANOVA, F(1,27) = 226.82, $P < 0.001$; autumn at low food: two-way ANOVA, F(1,13) = 495.75, $P < 0.001$; Fig. 2). The presence of predator (krill) did not have any significant effect on pellet production rates in any of the experiments at high food (summer: RBD ANOVA, F(1,50) = 3.15, $P > 0.05$; autumn: RBD ANOVA, F(1,27) = 0.04, $P > 0.05$; Fig. 2), but had a significant effect in the experiment on 25 October at low food (two-way ANOVA, F(1,13) = 13.63, $P < 0.01$; Fig. 2). However, in this last case krill caused a decrease of only 12% in the daily production of faecal pellets by copepods. No significant interactions between the factors day/night and absence/presence of predator were found (summer at high food: RBD ANOVA, F(1,50) = 0.91, $P > 0.05$; autumn at high food: RBD ANOVA, F(1,27) = 0.73, $P > 0.05$; autumn at low food: two-way ANOVA, F(1,13) = 1.19, $P > 0.05$).

The intensity of copepod feeding rhythms (i.e. night/day ratios of pellet production rates) were significantly different between seasonal photoperiods (two-way ANOVA, F(1,9) = 29.97, $P < 0.001$) and between food conditions (two-way ANOVA, F(1,9) = 62.58, $P < 0.001$). The night/day ratios of pellet production were 1.2–1.4 in summer at high prey concentration, 1.6–1.8 in autumn at high prey concentration, and 2.3–2.4 in autumn at low prey concentration (Fig. 2).

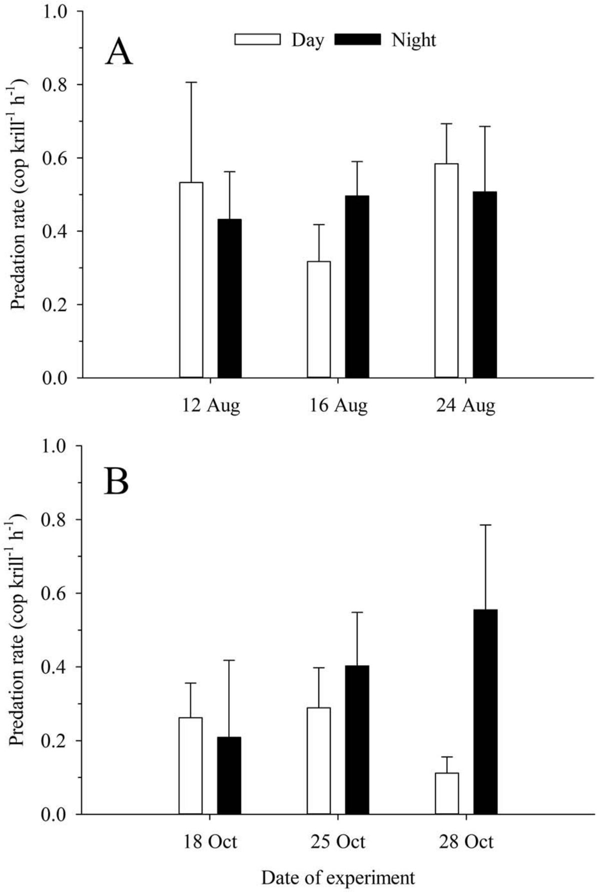

Fig. 1. Predation rates of the krill *Meganyctiphanes norvegica* (mean ± SE, n = 4–6) on the copepod *Centropages typicus* in experiments conducted in (A) summer (16 h:8 h day: night cycle) and (B) autumn (10 h:14 h day: night cycle).

DISCUSSION

Previous studies linked feeding rhythms of wild copepods to nocturnal forays into food-enriched upper layers during diel vertical migrations (Baars and Oosterhuis, 1984; Simard *et al.*, 1985; Besiktepe *et al.*, 2005). In our experiments, given the bottle sizes, we did not expect any relevant light-induced spatial heterogeneity in the distribution of the copepod prey *Oxyrrhis marina* (see Methods section) that could not be overcome by the swimming activity and the prey detection capability of *Centropages typicus* (Tiselius and Jonsson, 1990; Bundy *et al.*, 1993; Gonçalves and Kiørboe, 2015). Therefore, our study suggests that the rhythmic feeding behaviour of the copepod *C. typicus* might have an adaptive significance itself, which seems independent of migratory behaviour or changing food conditions (Head *et al.*, 1985; Durbin *et al.*, 1990). As evidenced in our experiments, such rhythms do not necessarily imply the complete cessation of feeding during the daytime (*sensu* Dagg *et al.* (1998)), but a lower

Table II: Sizes of copepods (Centropages typicus) and prey (Oxyrrhis marina), faecal pellet production rates of copepods and pellet volumes in incubations without predator ("Control") and with the predator Meganyctiphanes norvegica ("Predator"). Mean ± SE are shown. ESD: equivalent spherical diameter. n.d.: not determined

				Control		Predator	
Date of experiment	Time period	Copepod size (µm)	Prey size (ESD, µm^3)	Pellet production (pellets cop^{-1} h^{-1})	Pellet volume (µm^3)	Pellet production (pellets cop^{-1} h^{-1})	Pellet volume (µm^3)
12 Aug	Day	n.d.	16.2 ± 0.05	1.5 ± 0.10	183 462 ± 16 954	1.5 ± 0.07	191 441 ± 10 065
	Night	n.d.	16.0 ± 0.08	1.8 ± 0.18	203 021 ± 12 497	1.8 ± 0.07	223 268 ± 10 884
16 Aug	Day	1 182 ± 8.4	17.6 ± 0.03	1.3 ± 0.04	170 313 ± 12 730	1.0 ± 0.03	171 732 ± 12 338
	Night	1 168 ± 8.6	16.6 ± 0.01	1.3 ± 0.06	199 182 ± 8 950	1.4 ± 0.07	189 389 ± 8 527
24 Aug	Day	1 164 ± 14.3	16.5 ± 0.01	1.9 ± 0.04	288 239 ± 11 924	1.8 ± 0.06	268 105 ± 10 945
	Night	1 186 ± 10.3	16.4 ± 0.03	2.1 ± 0.05	298 799 ± 16 467	1.9 ± 0.06	311 400 ± 22 596
18 Oct	Day	1 256 ± 10.7	19.2 ± 0.03	1.9 ± 0.11	289 399 ± 12 370	1.9 ± 0.16	305 818 ± 13 861
	Night	1 225 ± 13.8	18.1 ± 0.02	2.2 ± 0.09	400 043 ± 22 237	2.3 ± 0.03	428 394 ± 24 822
25 Oct	Day	1 203 ± 10.9	20.4 ± 0.04	1.2 ± 0.04	220 836 ± 13 482	1.2 ± 0.06	189 468 ± 10 139
	Night	1 205 ± 14.6	19.3 ± 0.08	1.8 ± 0.07	336 411 ± 19 286	1.6 ± 0.03	331 894 ± 19 619
28 Oct	Day	1 203 ± 10.6	17.4 ± 0.04	1.7 ± 0.08	290 791 ± 18 121	1.5 ± 0.04	303 520 ± 22 506
	Night	1 212 ± 14.5	17.0 ± 0.02	1.8 ± 0.05	443 893 ± 24 626	1.9 ± 0.06	412 872 ± 22 900

foraging activity during daylight hours (Atkinson *et al.*, 1996; Olivares *et al.*, 2020).

The diel activity patterns of marine copepods can change upon the presence of predators (Ohman, 1988; Bollens and Frost, 1989; Bollens and Stearns, 1992). The krill *Meganyctiphanes norvegica* exert an important predatory pressure on copepod populations in the Northeast Atlantic (Beyer, 1992; Båmstedt and Karlson, 1998; Onsrud and Kaartvedt, 1998) and can affect their vertical migration patterns (Tarling *et al.*, 2002). Because the krill densities in our experiments were higher than typical average abundances in nature (Onsrud and Kaartvedt, 1998; Tarling *et al.*, 1998), we would expect the predator-induced responses of copepods to have been maximized. Still, we did not detect any large effect of krill presence on the feeding behaviour of copepods, either in diel feeding patterns or daily food intake. In all the cases the presence of krill barely affected the mean daily productions of copepod faecal pellets (from −8.7 to 12.2%), and such small differences proved to be statistically significant only in one out of the six experiments. Given the low variability among replicates in the faecal pellet production rates (median of coefficients of variation: 7.8%), any undetected effect of krill on copepod feeding activity was small. Thus, the non-lethal direct effects of predators on the feeding activity of marine copepods, including diel feeding rhythms, could be limited and/or depend on type of predator (Ohman, 1990).

In our study, *M. norvegica* showed predation rates that were highly variable among individuals. Such a flexible feeding behaviour has been previously observed in other experimental studies with *M. norvegica* predating on copepods (McClatchie, 1985; Båmstedt and Karlson, 1998; Agersted and Nielsen, 2016). We did not detect differences between day and night or between seasons in krill predation rates, but given the high variability of predation rates and the sample size in our experiments, we cannot discard that certain diel or seasonal differences in the feeding activity of krill could have been unnoticed (Torgersen, 2001). Our design was based on a power = 0.8 and standard deviations of 25–40% of the mean, but we observed a much higher variability than anticipated (median = 94% of the mean). It is also possible that the diurnal predation rates of *M. norvegica* could have been enhanced if copepods had been feeding on a more pigmented prey (e.g. phytoplankton) that increases copepod susceptibility to visual predation (Juhl *et al.*, 1996; Tsuda *et al.*, 1998), instead of an heterotrophic, not pigmented protist. Still, Abrahamsen *et al.* (2010) reported that *M. norvegica* might rely more on hydromechanical signals than vision to detect active prey like copepods. Actually, *M. norvegica* conduct diel vertical migrations (Onsrud and Kaartvedt, 1998; Onsrud *et al.*, 2004) and most encounters between *M. norvegica* and copepods might take place at night when visual predation is limited. Particularly in the Gullmar Fjord, populations of *M. norvegica* remain deeper during the daytime and ascend to layers above 30 m only at night (Spicer and Strömberg, 2002). Thus, copepods may find more advantageous to modify their diel antipredator feeding behaviour upon the presence of other visual predators like fish that occur in upper, more illuminated layers during daylight hours (Øresland and André, 2008).

We found that seasonal photoperiod and prey availability had a significant influence on the diel feeding behaviour of copepods. The feeding rhythms were less

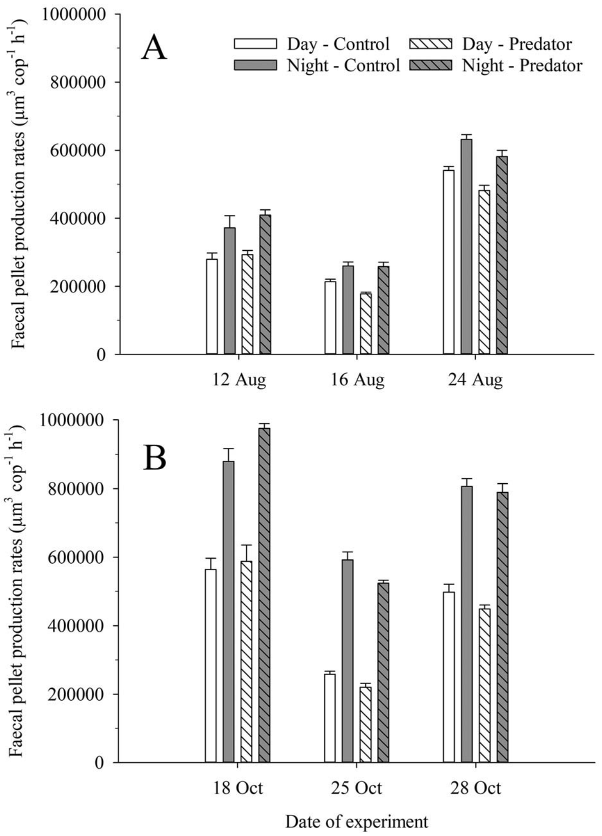

Fig. 2. Day and night faecal pellet production rates (mean ± SE, n = 4–6) of *Centropages typicus* feeding on *Oxyrrhis marina* in the absence of predator ("Control") and in the presence of the predator *Meganyctiphanes norvegica* ("Predator"). Data from experiments in (A) summer (16 h:8 h day: night cycle) and (B) autumn (10 h:14 h day: night cycle) are shown. Notice that *O. marina* concentrations were 1 ppm on 25 October, and 5.5–7.0 ppm in the other experiments.

pronounced in summer (16 h of light) than in autumn (10 h light). The diel rhythms of marine copepods are usually flexible over seasons (Båmstedt, 1984; Williams and Conway, 1984; Frost, 1988; Durbin *et al.*, 1995). Frost (1988) suggested that such variations between seasons are independent of prey availability, metabolic balance or thermal stratification, but driven by predation risk. In this regard, the seasonal photoperiod defines the relative time that copepods are exposed to a higher visual predation risk. As periods of higher predation vulnerability become relatively longer, copepods might diminish their antipredator behaviour to optimize the trade-off between eating and not being eaten (Lima and Bednekoff, 1999). Clearly, copepods must lower the intensity of their feeding rhythms when safe periods (i.e. dark periods) are too short for feeding to meet metabolic demands.

Regarding prey availability, the hunger/satiation hypothesis affirms that higher food availability normally results in larger amplitudes of vertical migration (Huntley and Brooks, 1982; Verheye and Field, 1992), which would enhance copepod feeding rhythms if food conditions in upper layers are better (Simard *et al.*, 1985; Peterson *et al.*, 1990; Besiktepe *et al.*, 2005). We found that the diel rhythms of *C. typicus* in the laboratory were more intense at low food concentrations. Low-food conditions decrease encounter rates with prey and copepods must swim for longer times and cover larger distances to feed (Saiz *et al.*, 1992), thus increasing their risk of being detected by predators (Uttieri *et al.*, 2013; Kiørboe *et al.*, 2014). Under these conditions, copepods would instead enhance their nocturnal feeding activity because foraging during the daylight hours would be too risky for them. We cannot strictly test this hypothesis since we only conducted one low-food experiment, but our findings are in agreement with those of Hassett and Blades-Eckelbarger (1995), who found that the diel feeding cycles of *Acartia tonsa* were more pronounced in the low-food treatment. In contrast, Calbet *et al.* (1999) reported that at lower food concentrations the diel feeding rhythms of *C. typicus* remained invariable and those of *A. grani* even vanished. Hence, the effect of food availability on copepod nocturnal feeding remains unclear and might be species-specific and/or depend on environmentally determined previous life history.

CONCLUSIONS

Our study is one of the few works that addressed direct effects of predators on copepod feeding rhythms using freely swimming predators instead of predator exudates. Still, we did not detect any large effect of the predator *Meganyctiphanes norvegica* on the diel feeding behaviour of the marine calanoid *Centropages typicus*, whereas effects of other factors such as seasonal photoperiod and prey availability emerged. Therefore, the non-lethal direct effects of predators on the feeding activity of marine copepods might not be as relevant as in freshwater zooplankton, and it will require further effort to assess their role in plankton trophic interactions in marine systems.

DATA ARCHIVING

Our data will be archived in a data repository after publication.

ACKNOWLEDGEMENTS

We would like to thank Ursula Schwarz for assisting with zooplankton sampling, Bengt Lundve for technical support and Kirti Ramesh, Ana Lopes and Theresa Haller for helping with phytoplankton culture maintenance. This study is a contribution of the Marine Zooplankton Ecology Group (2017 SGR 87). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

FUNDING

The research leading to these results received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730984, ASSEMBLE Plus project. The open access publication fee was covered by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). This study was framed within the project FERMI (CGL2014–59227-R, MINECO/AEI/FEDER, UE), and M.O. was supported financially by an FPU grant (FPU15/01747) from the Spanish Ministry of Education, Culture and Sport.

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