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

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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.

34 **Keywords**

35 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 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 & 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 $19\pm1^{\circ}\text{C}$ and 10 h: 14 h light:dark photoperiod (average PAR during the daytime: $90\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ for algae, $10\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ 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- μ m 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- μ m 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: $16\pm 1^\circ\text{C}$ and 13.5 h: 10.5 h light:dark cycle for *C. typicus*, and $19\pm 1^\circ\text{C}$ and 10 h: 14 h light:dark cycle for *P. grani*.

For laboratory-reared copepods, up to eleven 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 hours 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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.

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 2500L 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- μ m sieve and then vacuum-filtered through a 0.2- μ m 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 hours (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 μ m⁻³ and 0.221 pg C μ m⁻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 *Centropages 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 *Paracartia 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 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} $\mu\text{g } C_{\text{cop}}^{-1} \text{ h}^{-1}$, night: 105 ± 1.2 ng C_{prey} $\mu\text{g } C_{\text{cop}}^{-1} \text{ h}^{-1}$; 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 C_{prey} $\mu\text{g } C_{\text{cop}}^{-1} \text{ h}^{-1}$ 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 *Centropages typicus* and *Paracartia 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. $20 \mu\text{mol m}^{-2} \text{s}^{-1}$) 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

396 not for endogenous causes, which may bring concern for the use of cultured copepods in
397 experiments involving their diel feeding behaviour. The maintenance of diel rhythms in the first
398 laboratory-reared generation of *C. typicus* indicates epigenetic mechanisms that mediate the
399 non-genetic transmission of phenotypic traits (maternal effects). The evidence of ontogenetic
400 variations in diel feeding behaviour stands out the importance of age-related factors, such as
401 size-dependent vulnerability to predation, for the appearance of diel rhythms in copepods.
402 Finally, the lack of response to the exposure of predator exudates indicates that either predator-
403 induced responses of marine copepods might not involve chemical signalling, or factors other
404 than predation risk can act as driving factors of copepod feeding rhythms.

405 **Author contributions**

406 All the listed authors (Manuel Olivares, Albert Calbet, Enric Saiz) have contributed to the
407 experimental design, the field and laboratory work, the data analysis/interpretation, and the
408 manuscript preparation. All the listed authors have also approved the final version of this
409 manuscript.

410 **Disclosure of potential conflicts of interest**

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

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628 **Table captions**

629 **Table 1.** Size and concentration of copepods (*Centropages typicus*, *Paracartia grani*) and prey
630 (*Oxyrrhis marina*, *Rhodomonas salina*) in the feeding experiments. The volume of the bottles is
631 also provided. ESD: equivalent spherical diameter. N: nauplii. C: copepodites. Means $\pm$ SD are
632 shown

Figure captions

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)

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

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

Table 1. Size and concentration of copepods (*Centropages typicus*, *Paracartia grani*) and prey (*Oxyrrhis marina*, *Rhodomonas salina*) in the feeding experiments. The volume of the bottles is also provided. ESD: equivalent spherical diameter. N: nauplii. C: copepodites. Means $\pm$ SD are shown.

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)
Multigenerational rearing	<i>C. typicus</i>	F0	Females	977 $\pm$ 57	<i>O. marina</i>	16.8 $\pm$ 0.4	9.6 $\pm$ 0.4	33 $\pm$ 3	620
		F1-F11	Females	1032 $\pm$ 33	<i>O. marina</i>	17.1 $\pm$ 0.5	9.9 $\pm$ 0.4	32 $\pm$ 3	620
	<i>P. grani</i>	F0	Females	845 $\pm$ 2	<i>R. salina</i>	7.2 $\pm$ 0.02	4.7 $\pm$ 0.6	15 $\pm$ 1	320
		Fn	Females	1054 $\pm$ 2	<i>R. salina</i>	7.3 $\pm$ 0.03	4.6 $\pm$ 0.5	12 $\pm$ 3	320
Ontogeny	<i>C. typicus</i>	F1	NII-III	166 $\pm$ 14	<i>R. salina</i>	7.4 $\pm$ 0.20	3.6 $\pm$ 0.4	281 $\pm$ 46	72
		F1	NIV-V	224 $\pm$ 18	<i>R. salina</i>	7.4 $\pm$ 0.19	3.6 $\pm$ 0.4	171 $\pm$ 39	72
		F1	Males	964 $\pm$ 5	<i>O. marina</i>	16.7 $\pm$ 0.5	9.8 $\pm$ 0.4	38 $\pm$ 2	620
		F1	Females	1008 $\pm$ 7	<i>O. marina</i>	16.7 $\pm$ 0.5	9.7 $\pm$ 0.3	29 $\pm$ 2	620
	<i>P. grani</i>	Fn	NII-III	144 $\pm$ 2	<i>R. salina</i>	7.4 $\pm$ 0.10	2.3 $\pm$ 0.3	244 $\pm$ 44	72
		Fn	NIV-V	207 $\pm$ 0	<i>R. salina</i>	7.4 $\pm$ 0.09	2.3 $\pm$ 0.3	97 $\pm$ 28	72
		Fn	CII-III	573 $\pm$ 31	<i>R. salina</i>	7.0 $\pm$ 0.02	2.5 $\pm$ 0.3	56 $\pm$ 9	320
		Fn	CIV-V	803 $\pm$ 9	<i>R. salina</i>	7.3 $\pm$ 0.07	2.9 $\pm$ 0.2	57 $\pm$ 19	320
		Fn	Males	911 $\pm$ 10	<i>R. salina</i>	7.3 $\pm$ 0.04	4.6 $\pm$ 0.5	29 $\pm$ 5	320
		Fn	Females	1054 $\pm$ 2	<i>R. salina</i>	7.3 $\pm$ 0.03	4.6 $\pm$ 0.5	12 $\pm$ 3	320