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Microzooplankton grazers induce chain length plasticity in colonial diatoms

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Abstract

Many diatoms form long chains and the distribution of chain lengths within a species depends on several environmental factors, among them grazing risk. Larger grazers, such as copepods, efficiently handle and ingest even very long chains but are less efficient with individual cells, whereas most smaller grazers are unable to feed on chains exceeding a few cells in length. Copepod cues make several species shorten their chain length, and theory predicts that cues from small grazers should induce increased, but this remains to be tested, despite the importance of diatoms in marine food webs. Here, we expose three species of chain-forming diatoms, *Skeletonema marinoi*, *Chaetoceros affinis*, and *Thalassiosira rotula* to cues from various actively feeding micrograzers and record their response. The effect of grazer presence on chain length varies depending on both the type of grazer and the diatom species. For example, *S. marinoi* increased its chain length when exposed to grazing cues from the heterotrophic dinoflagellate *Gyrodinium dominans* and the ciliate *Euplotes* sp. but not to a copepod nauplii (*Temora longicornis*). *C. affinis* also responded to cues from grazing *G. dominans* by increasing chain length, and the response increased with exposure time. Finally, *T. rotula* did not respond to grazing cues from *G. dominans*, but rather inhibited the dinoflagellates' ability to feed, presumably through the release of chemical compounds. Our results suggest that some chainforming diatoms can sense and appropriately respond to fast-growing micrograzers, thus contributing to the success of diatoms in marine environments.

Zooplankton are often limited in their grazing by the size and shape of potential prey (Hansen et al. 1994; Kiørboe 2016; Ryderheim et al. 2022*b*). Thus, many phytoplankton form colonies of different shapes and sizes to protect themselves from grazing by moving outside the prey-size spectra of a certain grazer (Pančić and Kiørboe 2018). Grazers influence colony formation and size, both directly through handling and mechanical breakup of large colonies (Martin 1970; O'Connors et al. 1976; Ryderheim et al. 2022*b*), and indirectly through the release of chemical alarm cues (i.e., grazer cues) that induce colony size plasticity (Selander et al. 2019). Many colonial phytoplankton respond to such cues by altering shape and morphology. The prymnesiophyte *Phaeocystis globosa* form colonies in response to microzooplankton (20–200 μm) grazers (Jakobsen and Tang 2002; Long et al. 2007). In contrast, they break up colonies into smaller units or single cells in response to cues from larger grazers such as copepods (Long et al. 2007). Freshwater green algae of the genus *Scenedesmus* increase colony size in response to chemical cues from cladoceran grazers (Hessen and Van Donk 1993; Fisher et al. 2016; Zhu et al. 2016). Finally, many chain-forming diatoms respond to cues from copepod grazers by splitting up colonies into shorter chains or single cells (Bergkvist et al. 2012; Rigby and Selander 2021). These responses allow the phytoplankton to minimize grazing losses to the current grazer regime. Thus, the ability of colonial phytoplankters to appropriately respond to the current predator community is highly beneficial to phytoplankton fitness (Bjærke et al. 2015; Ryderheim et al. 2022*b*). In addition, how phytoplankton respond to predator cues may ultimately influence predator grazing behavior, and subsequently biogeochemical processes and

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Additional Supporting Information may be found in the online version of this article.

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the flow of energy in marine food webs (Lass and Spaak 2003; Pohnert et al. 2007; Pančić and Kiørboe 2018; Pinti et al. 2022; Kuhlisch et al. 2024).

How micrograzers influence colonial phytoplankton have been commonly studied in green algae and *Phaeocystis* spp. (e.g., Hessen and Van Donk 1993; Long et al. 2007), but there has been little focus on diatoms, despite their importance in marine food webs (Smetacek 1999; Assmy et al. 2013; Benoiston et al. 2017). Many diatom species form long chains and the occurrence of chain-forming relative to single-celled forms or species depend on several environmental factors, among them grazing risk (Bjærke et al. 2015; Kenitz et al. 2020). Larger copepods efficiently handle and ingest even long chains (> 200 μm), whereas some micrograzers are unable to feed on chains exceeding a few cells in length ($\sim$ 80 μm) (Ryderheim et al. 2022*b*). Thus, we hypothesize that cues from small grazers should induce increased chain lengths, as seen with colony formation in *Phaeocystis* spp., but this remains to be tested for colonial diatoms. Chain-forming diatoms are among the most abundant phytoplankton during spring blooms in temperate ecosystems and during these times the grazer biomass is dominated by heterotrophic and mixotrophic dinoflagellates and ciliates (Riebesell 1991; Tiselius and Kuylenstierna 1996). Thus, an improved understanding of how chain-forming diatoms defend themselves from fast growing micrograzers may advance our knowledge of the factors that allow diatoms to bloom.

In this study, we exposed three species of chain-forming diatoms (*Chaetoceros affinis*, *Skeletonema marinoi*, and *Thalassiosira rotula*) to various predatory cues. We hypothesize that micrograzer associated cues will induce increased chain lengths. *S. marinoi* was exposed to the heterotrophic dinoflagellate *Gyrodinium dominans*, the ciliate *Euplotes* sp., and nauplii of the copepod *Temora longicornis*, while *C. affinis* and *T. rotula* were exposed to *G. dominans*. We find that the effect of micrograzer presence on chain length varies depending on both the type of grazer and diatom species. *C. affinis* and *S. marinoi* respond by increasing chain length. In contrast, we saw no such response by *T. rotula*.

Materials and methods

Experimental organisms

The chain-forming diatoms *C. affinis* CCAP1010/27, *S. marinoi* R05AC and *T. rotula* CCAP1085/20 were acquired from the Culture Collection of Algae and Protozoa, CCAP, (*C. affinis*) or the GUMACC algal bank at the University of Gothenburg (*S. marinoi*, *T. rotula*), and grown non-axenic in L1 medium with added silica ($\sim$ 500 μmol L^{-1}), a salinity of 28 psu at 16°C, 14 : 10 h light : dark cycle, and $\sim$ 100 μmol photons m^{-2} s^{-1}. We chose these species because of their varied responses to predatory copepod cues, copepodamides. *S. marinoi* and *T. rotula* both respond to these by breaking up chains into single cells, while *C. affinis* does not (Rigby and Selander 2021). The individual cell size also differs among these species (Table 1). Diatoms were kept in exponential growth phase by continuous dilution with L1-media prior to use in the experiments.

Table 1. Chain-forming diatoms and the grazers which they were exposed to in the incubation experiments. Size measurements (equivalent spherical diameter – ESD, ±SD) are based on measurements of 20 individual cells from different chains assuming a cylindrical shape.

Diatom	Strain	Size (ESD, μm)	Grazing exposure
C. affinis	CCAP1010/27	17.5 ± 2.4	*G. dominans*
S. marinoi	R05AC	10.1 ± 1.7	*G. dominans*, *G. dominans* extract, *Euplotes* sp. *T. longicornis* nauplii
T. rotula	CCAP1085/20	20.9 ± 1.8	*G. dominans*

The heterotrophic dinoflagellate *G. dominans* ICM-ZOO-GD001 ($\sim$ 18 μm equivalent spherical diameter, ESD, Institut de Ciències del Mar, Spain) and the ciliate *Euplotes* sp. (body length $\sim$ 100 μm, isolated from a culture of the copepod *T. longicornis*) were kept in dim light at 16°C on a diet of *Rhodomonas salina* (*G. dominans*) or *Teleaulax amphioxeia* (*Euplotes* sp.). Nauplii of the copepod *T. longicornis* were acquired by harvesting eggs from our laboratory culture. Upon hatching, the nauplii were feed *R. salina* in excess, and grown to stage N2–N3 (body length $\sim$ 150 μm). Grazers were starved in filtered seawater for 24–48 h before being used in the incubation experiments.

Grazing cue exposure experiments

Exposure experiments were done in incubators that were made up of two 68-mL culture-tissue flasks separated by a 5-μm mesh (Supporting Information Fig. S1) that allowed exchange of chemical signals but prevented physical contact between organisms in the different chambers (Tang 2003; Selander et al. 2006). Diatoms were diluted with L1 + Si media (500 cells mL^{-1} for *T. rotula*; 1000 cells mL^{-1} for *C. affinis* and *S. marinoi*, except in the 144 h *C. affinis* experiment where the initial density was 500 cells mL^{-1}) and added to one chamber, and micrograzers (300–800 *G. dominans* mL^{-1}, 50 *Euplotes* sp. mL^{-1}, or 25 nauplii) and diatoms were added to the other (3–4 replicates per species). The grazer side contained roughly 2–3 times more diatom cells than the cue side to avoid starving the grazers, except in the *Euplotes* sp. experiments where the cell density was the same on both sides. We used different grazer-prey combinations (Table 1). *Euplotes* sp. was only combined with *S. marinoi* since even the single cells of *C. affinis* and *T. rotula* are outside the prey-size spectra of this grazer (Fenchel 1986). Copepod nauplii were only used in combination with *S. marinoi*. Incubators without grazers were used as controls (n = 3–4 per species). The incubators were attached to a rotating plankton wheel ($\sim$ 1 rpm) in such a way that both

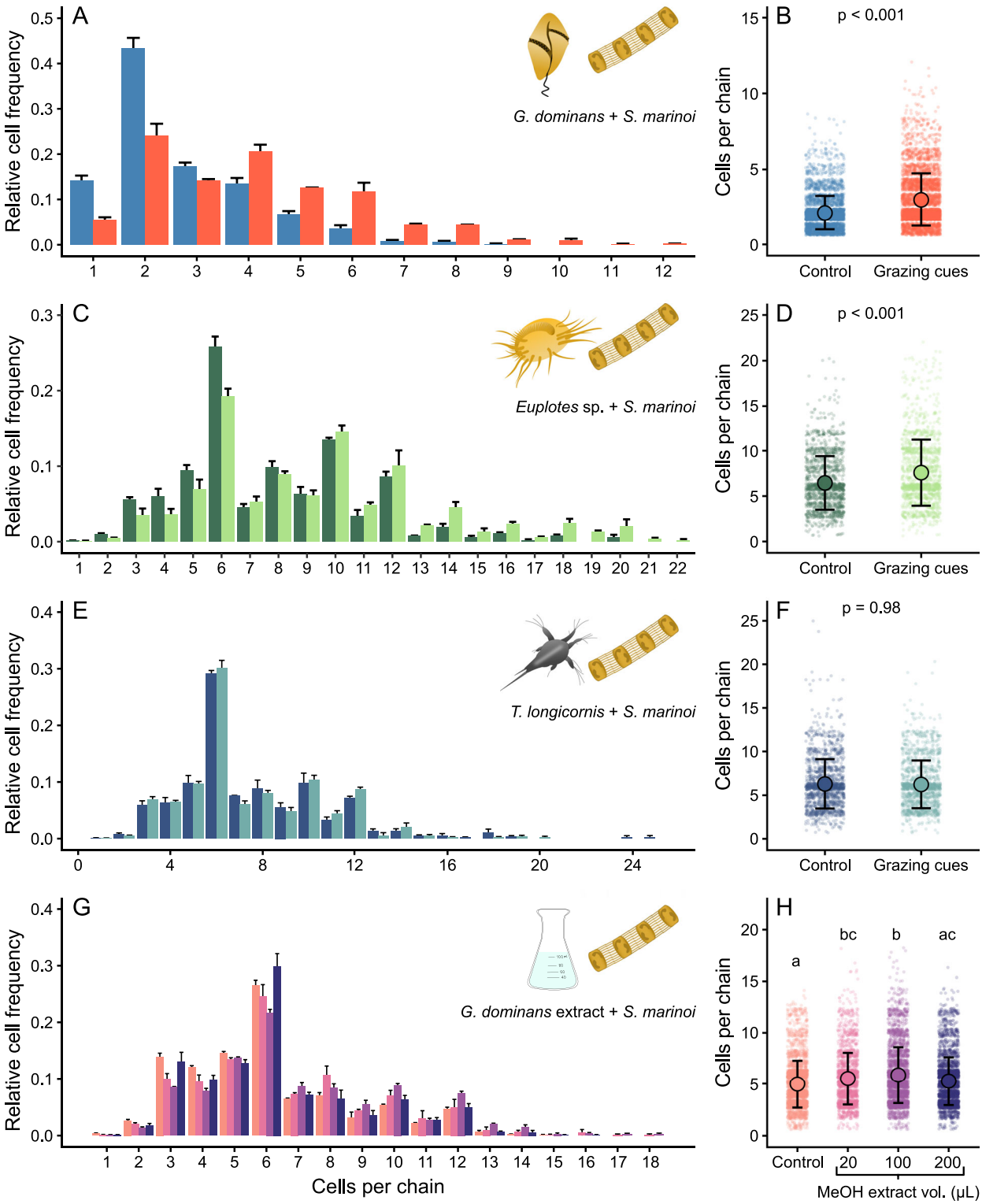

Fig. 1. Relative cell frequency distributions (i.e., number of cells × chain length/total amount of cells) for *S. marinoi* grown in the absence ("Control") or presence of grazing cues from *G. dominans* (**a**), *Euplotes* sp. (**c**), *T. longicornis* nauplii (**e**), or different amounts of MeOH extracted *G. dominans* (**g**). The bars show mean ($n = 3$), and the error bars are standard error. (**b**, **d**, **f**, **h**) Show the chain length count distributions for the two treatments. The large dots show mean chain length ± SD across all chains from 2 to 4 replicates and the small dots are the individual counts ($n = 1528$–3981 per treatment). The *p*-values indicate effect of the "Grazing cue" treatment. Lower case letters in (d) indicate differences among groups according to Tukey's HSD ($p < 0.05$).

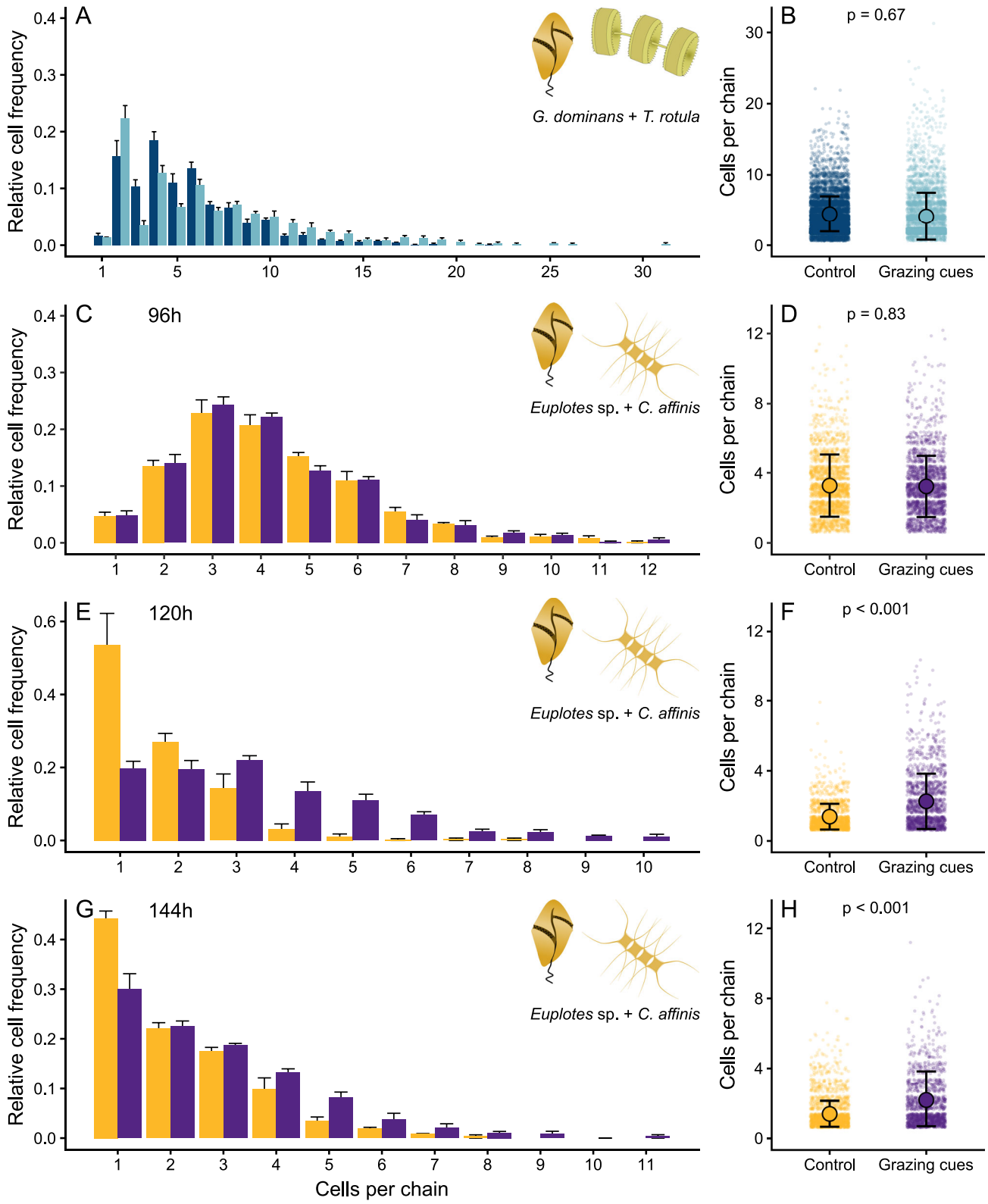

Fig. 2. Relative cell frequency distributions (i.e., number of cells × chain length/total amount of cells) for *T. rotula* (**a**) and *C. affinis* (**c**, **e**, **g**) in the diatoms growing in absence ("Control") and presence of grazing *G. dominans* ("Grazing cues"). The bars show mean ($n = 3$), and the error bars are standard error. (**b**, **d**, **f**, **h**) Show the chain length count distributions for the two treatments. The large dots show mean chain length ± SD across all chains from three replicates and the small dots are the individual counts ($n = 1567$–9154 per treatment). The *p*-values indicate the effect of the "Grazing cue" treatment.

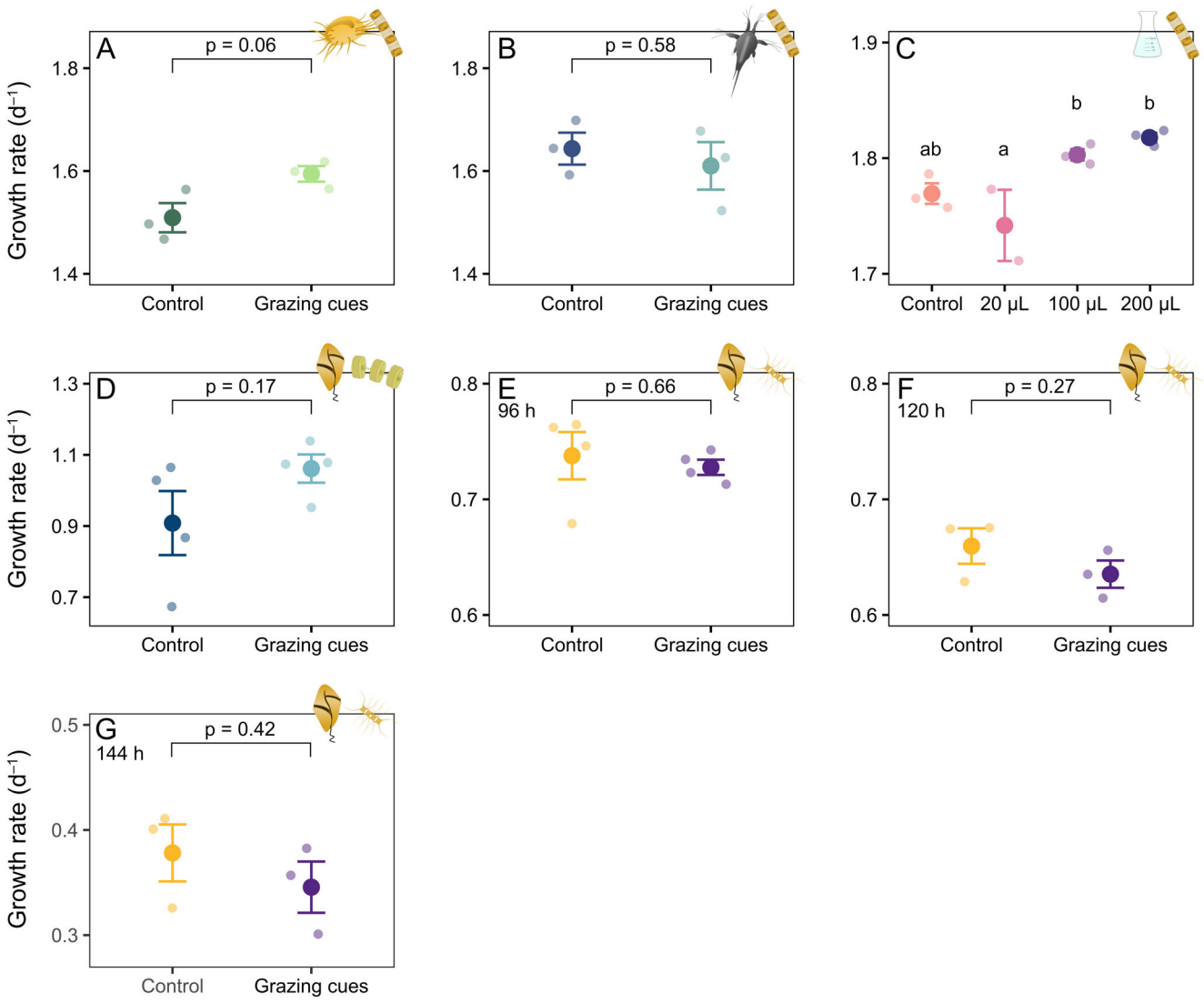

Fig. 3. Growth rates of diatoms when exposed to different grazing cues: *S. marinoi* exposed to *Euplotes* sp. (**a**), *T. longicornis* nauplii (**b**), and different amounts of MeOH-extracted *G. dominans* cues (**c**); *T. rotula* exposed to *G. dominans* (**d**); *C. affinis* exposed to *G. dominans* for different incubation lengths (**e**–**g**). The *p*-values indicate effect of the "Grazing cue" treatment. Large dots show mean growth rate ± SE ($n = 2$–4), and small dots are the individual replicates. Lower case letters in panel (c) indicate differences among groups according to Tukey's HSD ($p < 0.05$).

chambers received the same amount of light (~ 100 μmol photons m^{-2} s^{-1}). A small air bubble was left in the bottles to improve mixing and the transfer of cues across the mesh.

Incubations lasted for 72 h (*S. marinoi*–*T. longicornis*) or 96 h (all other combinations) h at 16°C. These exposure times have been sufficient to trigger defensive responses in other diatoms (Grønning and Kiørboe 2020; Olesen et al. 2022). With *C. affinis* we also performed two experiments with longer incubation times (120 h and 144 h), as this species have a lower growth rate compared with the other two. At the end of the incubations, a subsample from each chamber was fixed in Lugol's solution (final concentration 1%), and the cell density and chain length distribution (i.e., cells per chain) was determined in a Sedgewick Rafter chamber under the inverted microscope. At least 500 chains were counted and sized for each replicate. Micrograzers were enumerated in the same way, with at least 200 cells or all nauplii counted per replicate. Population growth rate (μ) for diatoms and grazers were estimated from the change in cell density assuming exponential growth using the formula

$$\mu = \frac{\ln D_t - lnD_0}{\Delta t},$$

where D_t and D_0 is the cell density at the end and start of the incubation, respectively, and Δt is the elapsed time in days.

Extraction of *G. dominans* cues

To extract grazer cues from *G. dominans*, we centrifuged 3 × 15 mL of well-fed stock culture at 2160 *g* for 8 min. The supernatant was removed, and the pellet resuspended in filtered sea water before being centrifuged again for 8 min. The supernatant was again removed, and the pellet was added to 1 mL methanol. We left the cells to extract for ~ 48 h before coating nine 68 mL glass bottles with 20, 100, or 200 μL of the extract ($n = 3$ per concentration), which corresponded to roughly 800, 4000, and 8000 *G. dominans* cells mL^{-1}, respectively. Three bottles were coated with pure methanol and used as controls. The methanol was evaporated under a stream of N_2 gas, and a suspension of *S. marinoi* (1000 cells mL^{-1}) was added to each bottle. The bottles were incubated for 72 h and analyzed as above.

Statistical analysis

Mean chain length was analyzed using a generalized linear mixed effects model with a Poisson distribution through the *lme4* R package (Bates et al. 2015). The different treatments were implemented as fixed effects, while replicates were included as random effects and nested under treatment. Growth rates were analyzed using one-way ANOVAs with a Tukey's HSD post hoc test using the *car* (Fox and Weisberg 2019) and *emmeans* (Lenth 2023) packages, respectively. All statistical tests were considered significant at the 0.05 level and are summarized in the Supporting Information (Table S1–S3).

Results

Grazing cue exposure experiments

The average chain length of *S. marinoi* increased by 41 ± 5% and 17% ± 6 (mean ± SE, $n = 3$) compared with the control when exposed to cues from *G. dominans* or *Euplotes* sp. feeding on *S. marinoi* (Fig. 1a–d). There was no increase in chain length in the treatment with feeding copepod nauplii (Fig. 1e,f). When exposed to the *G. dominans*-methanol extract, the average chain length increased 11 ± 5% and 18% ± 1 (mean ± SE, $n = 2$–4) in the 20 and 100 μL treatments, respectively, compared with the control (Fig. 1g,h). We saw a 6% ± 5 (mean ± SE, $n = 4$) increase with the largest volume added (200 μL), but this change was not statistically significant (Fig. 1h).

There was no increase in chain-length in *T. rotula* when exposed to *G. dominans* feeding on *T. rotula* (Fig. 2a,b), however, *G. dominans* decreased in density in these incubations by up to 70%. We initially found no increase in chain-length of *C. affinis* when exposed to feeding *G. dominans* for 96 h (Fig. 2c,d). However, in two subsequent experiments with longer incubation times we found a 63% ± 8 and a 22% ± 7 (mean ± SE, $n = 3$) increase in average chain-length compared with the control over 5 and 6 d incubations, respectively (Fig. 2e–h).

Diatom growth rates in the grazer exposed treatments did not significantly differ from the controls for any species or grazer combination (Fig. 3).

Discussion

Diatoms have evolved a wide variety of inducible defenses against larger sized predators (e.g., copepods), including shell thickening (Grønning and Kiørboe 2020; Ryderheim et al. 2022*a*), domoic-acid production (Olesen et al. 2022), chain-shortening (Bergkvist et al. 2012) and aggregation (Grønning and Kiørboe 2022). However, with the exception of aggregation, none of these seem to have negative effects on smaller protist grazers, such as dinoflagellates and ciliates (Olson and Lessard 2010; Pančić et al. 2019). Here, we demonstrate the ability of two chain-forming diatoms to sense and appropriately respond to cues from these smaller grazers by increasing colony size.

Response to micrograzer exposure

S. marinoi responds to cues from feeding *G. dominans* and *Euplotes* sp. by increasing chain length. *G. dominans* and *Euplotes* sp. seem restricted to feeding on shorter chains of *S. marinoi* (up to 6 cells or ~ 60 μm in length in *G. dominans*), suggesting that the increase in chain length is an adaptive and defensive response (Capriulo et al. 1988; Ryderheim et al. 2022*b*). Optimal predator prey ratios commonly recorded for these taxa, that is, 1 : 1 in heterotrophic dinoflagellates and 8 : 1 in ciliates (Hansen et al. 1994), further support the notion that longer chains provide protection from grazing. The effect of *G. dominans* cues extracted in methanol was weaker than those found in the incubation with live cells, perhaps due to degradation of cues. Grazer cues from copepods have a half-life below 3 h at 15°C (Selander et al. 2019). The *S. marinoi* cultures received cues only at the start of the incubations, whereas in the live grazer experiment they received them continuously. They are also not exposed to cues from grazed diatoms (i.e., "smell of death") (Hay 2009). Such intraspecific alarm cues have been found to induce defenses in other microalgae, for example, toxin production in *Alexandrium* spp., but results have been varied (Senft-Batoh et al. 2015; Griffin et al. 2019; Brown and Kubanek 2020). Nevertheless, the fact that *S. marinoi* responds to the extract suggests that at least part of the inducing effect is due to the presence of the dinoflagellate, and not the act of grazing. Further, simply receiving cues from dead algae would not give the diatoms information on whether increasing (against micrograzers) or decreasing (against copepods) chain length is the appropriate response. A similar argument can be made for any leftover *R. salina* in the extracted *G. dominans* suspension. Owing to declining *G. dominans* densities if left unfed throughout the incubations, we were unable to include a "grazer only" control. The activity in the crude methanol extract suggests that such extracts can be used as a starting

point for a bioassay guided fractionation approach to identify the cueing compound. Identification of the signal molecule would help provide mechanistic insights to the cause for increased chain formation.

T. longicornis nauplii feeding rates on *S. marinoi* decrease with increased chain length (Ryderheim et al. 2022*b*), but the diatom does not respond to cues from copepod nauplii. The reason may be that in our incubations, the nauplii increase in size (~ 1 stage per day) over time, thus, moving the upper limit of what they are able to feed on to include longer chains (Hansen et al. 1994; Kiørboe 2016). This likely also changes what an appropriate response would be from the diatoms (i.e., increasing in size against small nauplii and decreasing against larger). Moreover, copepod nauplii are typically accompanied by larger (copepodite and adult) developmental stages, and hence, reacting to nauplii cues may be counterproductive in natural settings.

In our initial experiment with *C. affinis*, we saw no increase in chain length in response to *G. dominans*. However, with longer exposure times we consistently found increased length. *C. affinis* have lower growth rates than the other two species (Fig. 3), and as the diatoms must grow to increase their chain length, the response time is likely longer in this species.

Grazer density decreased over time in the *T. rotula* experiments. *T. rotula* is known to produce allelochemicals with possible adverse effects on microzooplankton (Lavrentyev et al. 2015; Franzè et al. 2018). Dinoflagellates exposed to *T. rotula* showed negative growth rates inseparable from that of starved cells, even in the presence of alternative food normally sustaining growth (*R. salina*) (Supporting Information Fig. S2; Table S3). We were unable to observe whether the decrease was due to release of allelochemicals by the diatoms, or a toxic effect following ingestion, but clearance rates in the chain-length experiments were negative (Supporting Information Table S4), suggesting little or no grazing took place and that *T. rotula* somehow inhibits feeding in *G. dominans*. Our follow up experiment, although preliminary, seems to support this hypothesis, as *G. dominans* had access to *R. salina* in excess (Calbet et al. 2013) and, while still relatively unexplored, active prey selection do occur in protist grazers (Hansen and Calado 1999; Johnson et al. 2020). As such, the release of allelochemicals may be an alternative defense mechanism against microzooplankton in lieu of increasing in chain length (Flynn and Irigoien 2009; Franzè et al. 2018). However, this defense may be considered a "public good" since inhibiting or killing a grazer provides equal benefit to competing phytoplankton that do not produce the chemical. Defense can therefore not in general be considered the primary adaptive value of releasing allelochemicals (Jonsson et al. 2009; Driscoll et al. 2016). However, in non-motile phytoplankton, like diatoms, small-scale patches of monoclonal populations may persist for long enough that allelochemicals become partly privatized to benefit the individual cell and its offspring more than "cheaters" and competing species, and defense can therefore be the primary adaptive value of allelochemicals in diatoms (Ehrlich et al. 2022). The release of such chemicals as a potential defense mechanism against micrograzers is certainly an area of research that would benefit from increased attention.

One limitation of our study is the lack of feeding mode diversity. The *G. dominans* and *Euplotes* sp. used here feed by engulfment, i.e., they ingest the prey and digest it in an internal vacuole (Hansen 1992; Rode et al. 2022). Some heterotrophic dinoflagellates feed through a feeding tube, a peduncle, and some via an extracellular pallium (Hansen and Calado 1999). These feeding modes allow the dinoflagellates to feed on prey far exceeding their own size. Thus, one would imagine that these grazers would invoke a different response of the diatoms if they are not constrained to feeding on shorter chains as *G. dominans* and *Euplotes* sp. (Naustvoll 2000; García-Oliva and Wirtz 2022).

Trade-offs of chain formation

Predator-induced defenses are widespread in phytoplankton and are often assumed to come at a cost to the organism (Karban 2011), but these have seldom been quantified (Pančić and Kiørboe 2018). Defensive colony formation has in other colonial phytoplankton been shown to be associated with trade-offs, such as reduced growth rate (Wang et al. 2015; Zhu et al. 2016; Kapsetaki and West 2019), however, this has yet to be demonstrated in diatoms. We find no evidence of reduced growth with predator-induced chain length in our experiments. We note, however, that our experiments are done under non-limiting conditions. Some defense trade-offs are predicted to only become evident under resource limitation (Pančić and Kiørboe 2018), and this could apply also to chain formation since longer chains may experience reduced nutrient uptake (Pahlow et al. 1997). Our relatively high light levels during the incubations also reduce the effect of self-shading, and light has been found to influence colony related trade-offs in the green algae *Chlorella sorokiniana* (Kapsetaki and West 2019). Moreover, in a bidirectional inducible system like this one, where chains grow shorter in response to large grazers (copepods), but longer in response to small grazers (microzooplankton), it is hard to determine which is the induced stage. In grazer free environments chain forming diatoms still form chains.

Thus, the main trade-offs of chain formation may be increased encounter rates with larger predators or predators capable of feeding on long chains, rather than reduced cell division rate (Ryderheim et al. 2022*b*).

Ecosystem implications

The ability to respond to cues from both large and small grazers may have important implications for ecosystem dynamics, and particularly so in temperate systems where the spring bloom is often dominated by chain-forming diatoms (Riebesell 1991; Tiselius and Kuylenstierna 1996). As the

bloom is initiated, the diatoms will increase in density, but so will the microzooplankton as these often have growth rates similar to those of their prey (Hansen et al. 1997). Diatoms that can activate defenses against micrograzers, whether through longer chains or allelochemicals, may be able to escape predation and reach higher densities. As the bloom develops further and copepods start appearing, chains that break up into single cells in response to copepod cues will again have an advantage (Bergkvist et al. 2012; Ryderheim et al. 2022*b*). Thus, the ability of diatoms to harness a defense response that matches the current composition of the predator community may be one of the keys to their widespread success in marine ecosystems.

Data availability statement

Data are provided through Figshare (https://doi.org/10.6084/m9.figshare.23799879).

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Conflict of Interest

None declared.

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