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Thicker shells reduce copepod grazing on diatoms

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Significance statement

Diatoms are characterized by their silica shell, or frustule, that has been found to provide grazing protection against copepods. However, the underlying mechanism behind the reported reduced grazing mortality is still not known, mainly due to the frequent use of black-box incubation experiments. Here, we provide a mechanistic understanding through direct observation of copepod-diatom interaction of how the defense works: not only are diatoms with thicker shells more frequently rejected after being captured, but also take longer for the copepods to ingest, and thus, limit copepod ingestion. Both of these mechanisms have implications for diatom life history and the potential ecosystem services they provide.

Data availability: The data is available through the Dryad data repository at https://doi.org/10.5061/dryad.c59zw3r8z

Abstract

The diatom frustule provides partial protection against copepod grazing. Whether the defense is due to the cells being de-selected or handled for so long that the grazers lose time for foraging is unknown. The mechanism has implications for the population dynamics of both defended and co-occurring, undefended nutrient competitors. We use video-observations to demonstrate that thick-shelled diatoms captured by the copepod *Temora longicornis* were rejected more frequently than thin-shelled diatoms, irrespective of cell size. The thick-shelled cells of the larger diatoms were handled for much longer, and the time spent handling these limits the consumption of phytoplankton. This may be why many diatoms, even in the presence of dense grazer populations, reach bloom concentrations, and thus, facilitate aggregation and mass sedimentation. This has implications for both carbon sequestration and for securing a large population of cells at depth ready to colonize the pelagic, when growth conditions again become favorable.

Keywords: silica shells, defense mechanisms, diatoms, copepods, prey selection, grazing

Introduction

Diatoms account for about 40% of global ocean primary production (Falkowski et al. 1998), they are a significant source for the pelagic grazing food chain that supports higher trophic levels (Sommer et al. 2002), and they are a quantitatively important component of the biological carbon pump that sequesters carbon (Smetacek 1999; Benoiston et al. 2017). Their characteristic silica shell has many hypothesized functions that may govern both the production and the fate of diatoms and, hence, their important functions in marine ecosystems. For example, the siliceous shell may increase nutrient uptake (Mitchell et al. 2013), facilitate light harvesting (Romann et al. 2015), protect against UV radiation (Aguirre et al. 2018), and provide grazer defense (Smetacek 2001; Hamm et al. 2003). The latter role has been suggested based on the unparalleled strength of the diatom shell (Hamm et al. 2003; Aitken et al. 2016)

and has recently been demonstrated experimentally: the grazing mortality due to copepods generally varies inversely with the silica content of the cell across and within diatom species (Liu et al. 2016; Pančić et al. 2019). Diatoms may further thicken their shell in the presence of grazer cues, thus harnessing the defense when needed albeit at the cost of a reduced cell division rate (Pondaven et al. 2007; Grønning and Kiørboe 2020). These properties of diatoms and their shell are key to their evolutionary success and prominent role in the global ocean (Benoiston et al. 2017).

Krill and copepods, probably the most important grazers on diatoms, have silicified 'teeth' that should be well suited to crack the diatom shell if not too thick and strong (Michels et al. 2012). Reduced grazing on thick-shelled cells has been recorded in black box incubation experiments (Liu et al. 2016; Pančić et al. 2019), but whether the reduction is due to the cells becoming un-breakable, are de-selected, or are handled for so long that the grazers lose time for foraging, is unknown. The defense mechanism has implications for the population dynamics, both for the defended diatoms and for their nutrient competitors and, hence, the structure of phytoplankton communities and the fate of primary production. For example, de-selection reduces grazing pressure on defended cells, while longer handling may decrease ingestion also of other cells. In this study, we open the black box through direct observations of individual copepod-diatom interactions to reveal the mechanism of defense. We demonstrate that cells with thicker shells are generally handled for a longer time and rejected with a greater likelihood than thin-shelled cells, accounting for the lower grazing on heavily armored diatoms.

Materials and methods

Phytoplankton cultures

The diatoms *Cyclotella cryptica* (CCMP336, NCMA Bigelow Laboratory, Maine, USA), *Ditylum brightwellii*, and *Thalassiosira weissflogii* (both unknown strains, DTU Aqua,

Denmark) were grown in B1 medium with silica (~500 µM) at salinity 30 and temperature 16 °C. To acquire cells with different silica contents, we grew cultures in high light (HL, 120 µmol photons m^{-2} s^{-1}) or low light (LL, 15 µmol photons m^{-2} s^{-1}) at either 12:12 or 24:0 light:dark cycles because slower growing, light-limited cells grow thicker shells (Liu et al. 2016; Pančić et al. 2019). The cultures were kept in exponential growth phase by dilution with fresh media every few days.

Cell sizes and densities were monitored with a Multisizer 4e Coulter Counter (Beckman Coulter, California, USA), and exponential growth rates were calculated from temporal variations in biovolume (µm^3 mL^{-1}). The biogenic silica content of the diatoms was quantified as described in Grønning & Kiørboe (2020). Silica content is expressed per cell volume as the strength of the shell scales approximately with silica per volume, rather than with silica per surface area (Pančić et al. 2019).

Growth rates and silica contents of the diatoms used for experiments varied as expected: cells grown in the low light treatments had consistently lower growth rates and higher silica contents than cultures grown in high light (Table 1). Cell sizes were similar between treatments except for *C. cryptica*, which were smaller in the low light treatment (Table 1).

Copepod foraging behavior

We used the feeding-current feeding copepod *Temora longicornis* as model copepod grazer. These copepods vibrate their feeding appendages to generate a feeding current from which they harvest prey (Kiørboe 2011). Copepods were taken from a continuous culture, where they are maintained on a phytoplankton diet of *Rhodomonas salina, T. weissflogii, Heterocapsa triquetra*, and *Oxyrrhis marina.*

We filmed the foraging behavior of adult females that were glued to a human hair by their dorsal surface using cyanoacrylate-based super glue. The copepods were acclimated overnight

in filtered seawater and in darkness. The following day, the tethered copepod was attached to a micromanipulator and submerged in a 10×10×10 cm^3 aquarium. Prey cells were added (300 cells mL^{-1}) and kept suspended by a slowly rotating magnetic stirrer. Four 3-minute sequences of copepod foraging were immediately recorded at 50 fps using a Phantom V210 High-speed camera (Vision Research, New Jersey, USA). The tethered copepod was then transferred to an aquarium with the alternate prey treatment (HL or LL) and another four sequences were recorded. The camera was equipped with lenses to provide a field of view of 1.3×1.0 mm^2. Collimated light was provided by an infrared lamp shining through the aquarium directly towards the camera. Four (*C. cryptica*) or five (*T. weissflogii*, *D. brightwellii*) copepods were used for each prey species. The videos were analyzed for quantification of the number of cell captures, handling times (only *D. brightwellii* in this specific set-up), and the fraction of captured cells that were rejected by the copepod. In addition, we recorded captures of individual cells at a higher frame rate (1000 fps) and magnification (1.7×1.3 mm^2) to better quantify handling time for ingested prey (see Video S1 for example). These handling time recordings were done either the day after the foraging experiments (*C. cryptica*) using the same copepods, or at a separate time (*D. brightwellii*, *T. weissflogii*) using freshly tethered copepods.

In an additional experiment we tested the effect of food saturation and prey density on the efficiency of the defense. Copepods were glued as described above and fed *T. weissflogii* grown in low light conditions overnight at a concentration of 1000 cells mL^{-1}. The next day we fed the copepods *T. weissflogii* grown in low- or high light at 150 or 1000 cells mL^{-1} while observing their behavior as described above. All raw data are available in Grønning et al. (2022).

Statistics

To analyze the effect of cellular silica content on the fraction of rejected cells, we fitted a mixed-effects logistic regression to the data using the R package *lme4* (Bates et al. 2015). 'Treatment' (HL and LL grown cells) and 'Sequence' (to examine whether there was a temporal effect) were fixed effects and the individual copepods the random effect. The model was weighted by the amount of captures. The error term has a binomial distribution, and the random copepod intercept was assumed to be normally distributed. The best fitting model according to the Akaike information criterion (AIC) was used and validated by visual inspection of the residual plots. The random effect-variance component was close to zero for *C. cryptica* and *T. weissflogii* data, but was kept in the model to incorporate the dependency of the response variable on the copepods.

Results

Foraging behavior

The copepods detected and captured cells irrespective of their silica content. However, the fraction of captured cells that were rejected by the copepods was significantly higher for thick-shelled compared to thin-shelled cells in all three diatom species (Fig. 1a–c). In addition, cells of larger species were rejected more frequently than those of smaller species, particularly evident for thick-shelled cells.

Rejection frequency of *T. weissflogii* was similarly higher for the high silica treatment in the two experiments, where copepods were offered food prior to filming (Fig. 1d–e). Overall, cells were more frequently rejected in the high cell-density treatment (Fig. 1d–e), but the increased chance of rejection compared to the low silica treatment was similar (Odds ratios = 3.27 and 3.20 in the 150 and 1000 cells mL^{-1} experiments, respectively). Thus, prey density and saturation had no impact on prey selectivity.

There was generally no temporal effect ('Sequence') on the fraction of rejected cells, but in the high concentration *T. weissflogii* experiments (1000 cells mL^{-1}), the chance of rejection slightly decreased over the course of the four video sequences (mixed effects logistic regression, $z = -2.3$, $p = 0.02$).

Prey handling time

Captured cells were rejected or ingested only after being handled – 'examined' – by the feeding appendages. Some rejected cells were actively pushed away by the feeding appendages (Video S2), but the majority were just let go (Video S3). These more passive rejections were distinguishable from the interactions where the cell was lost by the copepod during the capture process.

The handling times of rejected *D. brightwellii* cells were shorter than those of ingested cells and independent of silica content (Fig. 2). In contrast, handling times of ingested cells were significantly longer for thick-shelled than for thin-shelled cells in *D. brightwellii* and *T. weissflogii*, while in the small *C. cryptica*, handling times were short and independent of silica content (Fig. 3). In addition, handling times of ingested cells increase with cell size, particularly evident for thick-shelled cells (Fig. 3).

Discussion

Handling and selection of captured diatoms

Feeding-current feeding copepods perceive particles that arrive in the feeding current individually as the particles come near the mechanoreceptory setae on the feeding appendages, which elicits a capture response (Koehl and Strickler 1981; Videos S1–3). Copepods are known to be selective feeders, but prey selection appears to happen post capture while the prey is being handled by the feeding appendages, as demonstrated here for diatoms with different silica content, and elsewhere for dinoflagellates with different degrees of toxicity (Ryderheim et al.

2021) or bioluminescence (Prevett et al. 2019). The feeding appendages are equipped with both mechano- and chemosensors (Hallberg and Skog 2011) and both sensory modalities are probably involved in prey selection. However, it is unclear how the copepod assesses the silica content or strength of the shell of diatoms.

Cells with high silica content are actively rejected by the copepods, particularly the larger cells. Small cells may be swallowed intact, while larger cells may need to be crushed before they can be ingested. The mouth width of the copepod *Oithona davisae* is about 10 μm and it has a prey size spectrum similar to that of *T. longicornis*, despite its smaller size (Saiz et al. 2014; Gonçalves et al. 2014). Thus, the size-threshold for swallowing/crushing cells before ingestion may be around 10 μm, hence accounting for the difference in the degree of selectivity and handling times between small and large prey in our experiments. This could also, at least in part, explain the recent findings of Xu et al. (2021) who reported that increased silica content in the diatom *Amphora coffeaefornis* had no defensive benefit, but rather the opposite, as copepods had higher ingestion rates on cells with increased silica content. The size of *A. coeffeaefornis* in their experiments was just ~8 μm ESD (it is also elongated in shape, i.e., the actual width of the cell is <8 μm), and in our experiments we clearly see a decrease in the efficiency of the defense with decreasing cell size. Larger copepods than *T. longicornis* may be able to feed on larger cells without crushing them, but larger copepods are found in areas where phytoplankton cells are also generally larger, i.e., in sub-polar and polar regions (Barton et al. 2013; Brun et al. 2016), and hence our findings may apply more generally.

The prey densities used in our study are within the natural range, e.g., as found during spring blooms in the North Sea (Riebesell 1991). However, prey density did not impact the selectivity of the copepods. While both cells with high and low silica were more frequently rejected in the high cell-density treatment, the increased chance of rejection due to thicker shells remained similar. Thus, while saturated prey conditions decreases the grazing mortality for diatoms that

195 do not invest in the costly defense (Pančić et al. 2019; Grønning and Kiørboe 2020), it also
196 increases the fitness of the ones that do. Hence, it will still be a worthwhile investment should
197 grazing pressure be sufficiently high.

198 The handling times of thick-shelled cells were up to 50% longer than that of thin-shelled cells
199 and therefore possibly limiting ingestion rates. Thus, estimating the maximum possible number
200 of thick-shelled cells consumed per time as 1/handling time, and estimating cellular carbon
201 contents from cell size (Menden-Deuer and Lessard 2000) yields maximum ingestion rates of
202 10–15 μg carbon female^{-1} d^{-1} for the three prey species. This is similar to maximum
203 consumption rates measured in incubation experiments for *T. longicornis* females (Franco-
204 Santos et al. 2018). However, this estimate disregards time spent handling cells that are
205 subsequently discarded, and since up to four out of five defended cells are discarded for every
206 cell consumed, this may decrease the maximum possible number of cells consumed, even
207 though discarded cells are handled faster than those consumed. Diatoms in nature typically
208 grow at average light intensities similar to, or less than our low-light treatment and they may
209 further thicken their shells if copepods are present (Grønning and Kiørboe 2020), thus reducing
210 consumption rates. Limitation by other nutrients may also lead to slower growth and
211 consequently thicker shells. Thus, some iron-limited Antarctic regions are dominated by
212 particularly thick-shelled diatoms, such as *Fragilariopsis kerguelensis* that contain 3 times
213 more silica than typical diatoms (Quéguiner et al. 1997; Smetacek 1999). Such thick-shelled,
214 highly defended diatoms decreases the grazing pressure on not only defended diatoms, but also
215 on undefended diatoms as well as on other phytoplankton due to the long handling times and
216 time spent handling rejected cells. The effect is instantaneous but may be exaggerated in the
217 longer term due to reduced copepod consumption and consequent propagation rates.

The reduction in consumption rate in the presence of defended diatoms applies not only at saturating prey concentrations, but at all prey concentrations, as evident from the Holling type II functional response in consumption rate (I) to prey concentration (C):

$$I = \frac{\beta C}{1 + \beta C \tau}, \qquad \text{Eq. 1}$$

where τ is the prey handling time and β the maximum clearance rate (i.e., the clearance rate at very low prey concentrations). At high prey concentration, the consumption rate is the inverse of the handling time ($I = 1/\tau$), and the effect of defense on consumption rate the highest. At lower prey concentrations, the effect decreases, cf. eq. 1.

The evolutionary arms race

Diatoms evolved ~250 million years ago more or less concurrent with the time that copepods evolved from benthic ancestors and colonized the pelagic. While the diatom frustule has many hypothesized advantages, ranging from viral protection to enhanced light harvesting and nutrient acquisition, the evolution of the frustule may have been partly the result of selection for grazer protection. Krill and copepods, in turn, have adapted to utilize this abundant food source by the evolution of siliceous 'teeth' that allows the grazer to crush diatoms that are not too thick-shelled. The silica-reinforced teeth of copepods may thus have evolved in an 'arms-race' response and may partly explain the dominance of copepods and krill in diatom grazing (Smetacek 2001). The need to crush larger diatom cells before ingestion may increase wear and tear on the copepods' teeth, which eventually may lead to an inability to forage on diatoms (Smetacek 2012; Michels et al. 2012). Both krill and copepods counteract this by frequent molting, where the exoskeleton including the teeth are renewed. Krill molt throughout their life cycle, while copepods stop molting when they reach adulthood. Thus, the ability of copepods, but not krill, to actively de-select heavily armored diatoms and reduce wear and tear may be an important component of this evolutionary arms race. Because late stage copepodites typically

dominate the biomass of copepods, the selective feeding of copepods, in particular, may feed back on the evolution of diatom defenses.

Implications for the fate of diatoms

Diatoms play a key role in the biological carbon pump (Smetacek 1999) and the degree of defense by shell-thickening may have implications for the fate of diatoms. Weakly defended diatoms may be consumed by copepods and thus enter the grazing food chain. Copepod fecal pellets sink or are re-mineralized in the upper ocean, depending on their size, ballasting, and consequent sinking speed (Stamieszkin et al. 2015). Some species of diatoms form dense blooms, mainly during vernal temperature stratification in temperate and arctic seas or in other 'new', nutrient rich ephemeral habitats, and such blooms are typically terminated by mass sedimentation rather than by grazing (Assmy et al. 2013). This is often ascribed to the time-lag in the numerical response of copepod grazers in neritic temperate systems, but it also applies to diatom blooms in open water systems where overwintering *Calanus* spp. ascend *en masse* to the surface simultaneously with the developing bloom (Dünweber et al. 2010) or where krill are present year round. The limited copepod grazing pressure on phytoplankton due to the presence of defended diatoms may allow these blooms to develop, and the consequently high bloom concentrations may facilitate aggregation and subsequent mass sedimentation. This, in turn, secures a large seed population at depth or in the sediment from which cells may colonize the pelagic when growth conditions again are favorable.

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Table 1. Summary of diatom prey used in the foraging and handling time (HT) experiments. Foraging experiments were carried out with multiple cell densities (noted in brackets as cells mL^{-1}). Cc: *C. cryptica*, Tw: *T. weissflogii*, Db: *D. brightwellii*. HL: high light, LL: low light. ESD: equivalent spherical diameter as measured by particle counter. Values for silica are means ± SD (n = 2–3).

Species	Exp. type	Treatment	L:D cycle	ESD (µm)	Growth (d^{-1})	Silica (fmol $µm^{-3}$)
Cc	Foraging (300 mL^{-1}), HT	HL	24:0	11.25	0.71	1.19 ± 0.09
Cc		LL		9.52	0.29	5.35 ± 0.21
Tw	Foraging (300 mL^{-1})	HL	12:12	14.66	0.41	0.62 ± 0.15
Tw		LL		15.12	0.29	0.84 ± 0.04
Tw	HT	HL	24:0	14.98	0.44	0.44 ± 0.10
Tw		LL		14.68	0.17	1.18 ± 0.01
Tw	Foraging (150 mL^{-1})	HL	24:0	13.08	0.29	0.46 ± 0.03
Tw		LL		13.56	0.17	0.66 ± 0.03
Tw	Foraging (1000 mL^{-1})	HL	24:0	13.12	0.45	0.44 ± 0.06
Tw		LL		13.25	0.32	0.71 ± 0.06
Db	Foraging (300 mL^{-1})	HL	12:12	22.99	0.20	0.69 ± 0.01
Db		LL		23.77	0.11	0.86 ± 0.01
Db	HT	HL	24:0	26.97	0.22	0.46 ± 0.01
Db		LL		28.05	0.06	0.74 ± 0.01

272 Figure legends

273 Figure 1. Fraction of caught cells that were rejected by the individual copepod in three diatom
274 species (a) *C. cryptica*, (b, d–e) *T. weissflogii*, and (c) *D. brightwellii*. Data in (a–c) are from
275 the experiments with starved copepods and 300 cells mL^{-1}, while (d) and (e) are the *T.*
276 *weissflogii* experiments with pre-fed copepods and 150 and 1000 cells mL^{-1}, respectively. Red
277 and blue bars denote diatom cultures with low and high silica content, respectively. *P*-values
278 indicate significant effect of the treatment on rejection frequency. Odds ratios with 95%
279 confidence intervals: (a) Cc: 1.51 [1.10, 2.07], (b) Tw: 4.57 [3.57, 5.90], (c) Db: 3.26 [2.39,
280 4.47], (d) Tw150: 3.27 [2.50, 4.28], (e) Tw1000: 3.20 [2.76, 3.70]. Error bars show 95% Wilson
281 Score Interval (n = 35–535).

282 Fig 2. Prey-handling times derived from low-speed (50 fps) videos for (a) ingestions and (b)
283 rejections of *D. brightwellii* with cells of low (red) and high (blue) silica content. The data are
284 compiled from observations of several copepods per treatment. Asterisks indicate significant
285 differences between treatments according to one-way ANOVA (Ingestions: $F_{1,92} = 25.70$, $p <$
286 0.001; Rejections: $F_{1,98} = 0.001$, $p > 0.05$).

287 Figure 3. Copepod prey-handling time for ingested prey derived from high-speed videos (1000
288 fps) of (a) *C. cryptica,* (b) *T. weissflogii,* and (c) *D. brightwellii* for cells with low (red) and
289 high (blue) silica content. Cell sizes increase from panel (a) to (c). The data are compiled from
290 observations of several copepods per species and treatment. Large circles show means ± SD
291 and small circles are the individual data points. Asterisks indicate significant differences
292 between treatments according to one-way ANOVA. Cc: $F_{1,34} = 0.64$, $p = 0.43$; Tw: $F_{1,25} =$
293 15.77, $p < 0.001$; Db: $F_{1,35} = 11.67$, $p = 0.002$.

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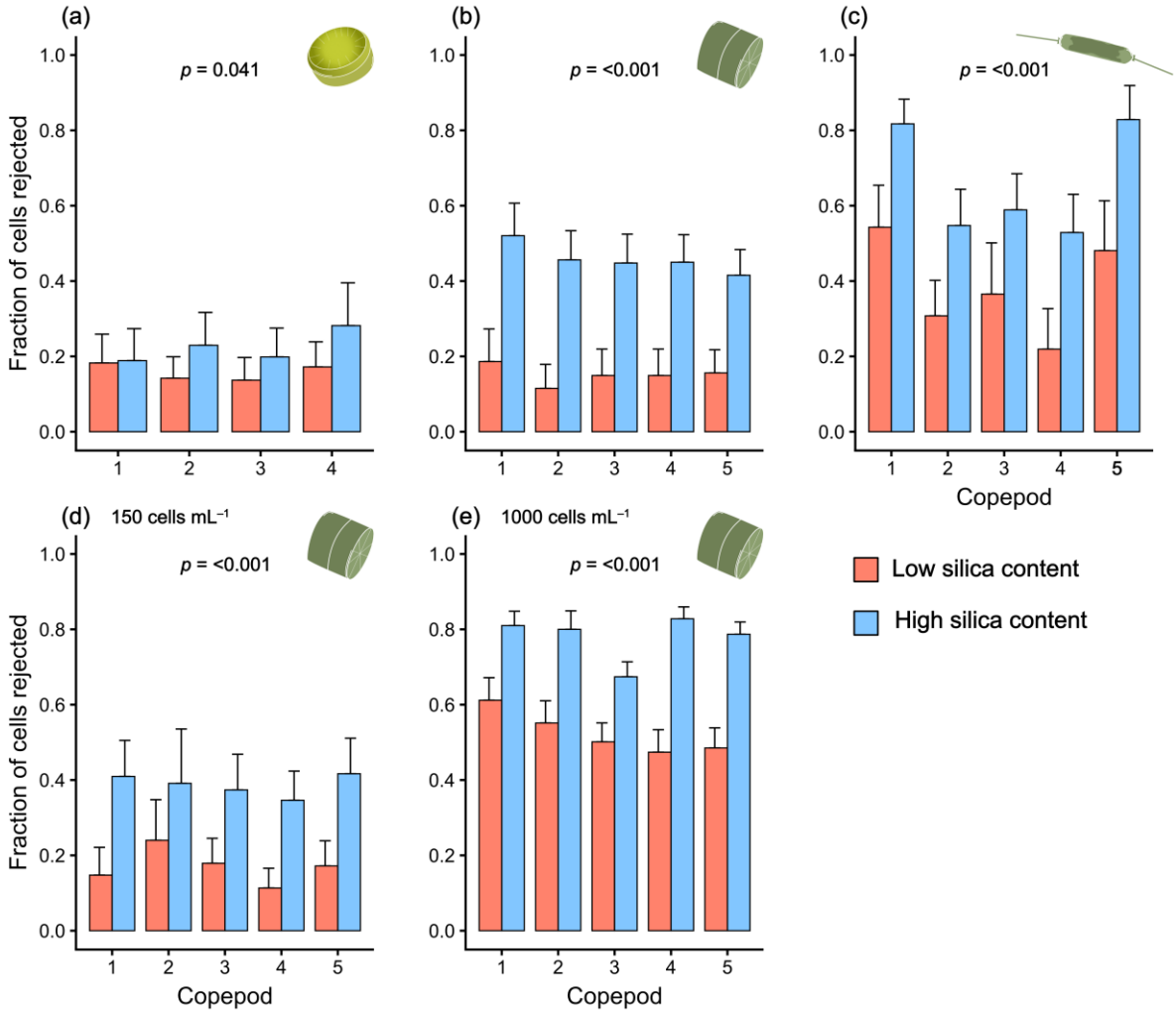
400 Figure 1.



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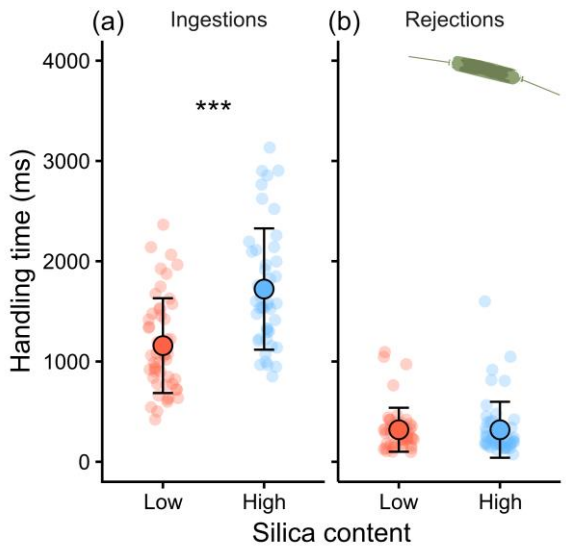
403 Figure 2.



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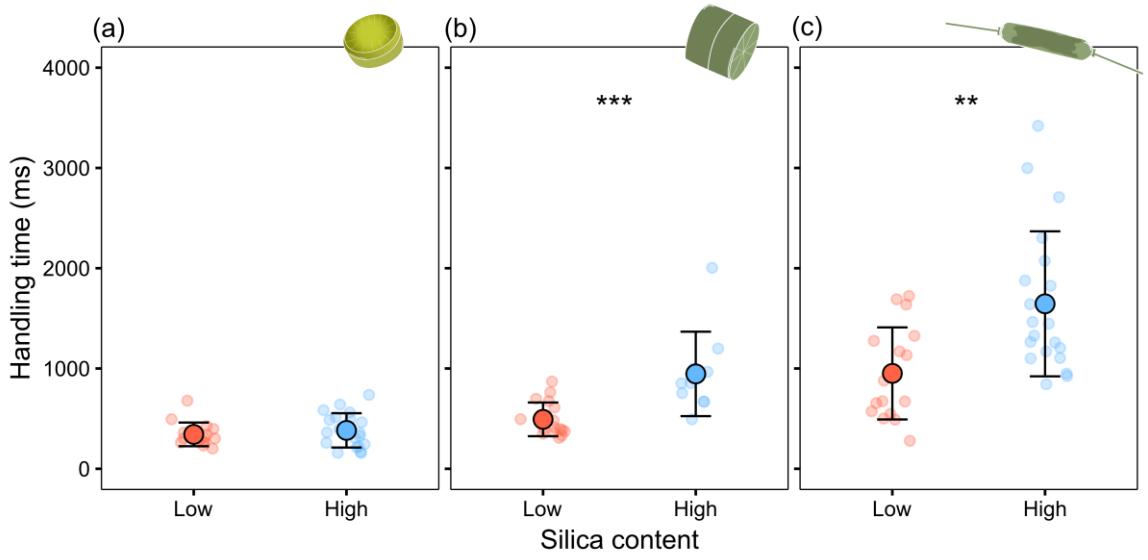
406 Figure 3.



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