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1 **Costs and benefits of predator-induced defence in a toxic diatom**

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14

15 We declare no competing interests.

16 ***Abstract***

17 Phytoplankton employ a variety of defence mechanisms against predation, including  
18 production of toxins. Domoic acid (DA) production by the diatom *Pseudo-nitzschia* spp. is  
19 induced by the presence of predators and is considered to provide defence benefits, but the  
20 evidence is circumstantial. We exposed eight different strains of *P. seriata* to chemical cues  
21 from copepods and examined the costs and the benefits of toxin production. The magnitude  
22 of the induced toxin response was highly variable among strains, while the costs in terms of  
23 growth reduction per DA cell quota were similar and the trade-off thus consistent. We found  
24 two components of the defence in induced cells: (1) a ‘private good’ in terms of elevated  
25 rejection of captured cells, and (2) a ‘public good’ facilitated by a reduction in copepod  
26 feeding activity. Induced cells were more frequently rejected by copepods and rejections  
27 were directly correlated with DA cell quota and independent of access to other food items. In  
28 contrast, the public-good effect was diminished by the presence of alternative prey suggesting  
29 that it does not play a major role in bloom formation and that its evolution is closely  
30 associated with the grazing-deterrent private good.

31

32 Keywords: Diatom defence, copepods, marine chemical interactions, defensive benefit, trade-  
33 offs.

## 34 ***Introduction***

35 The herbivory pressure in pelagic ecosystems is intense, with up to three times higher fraction  
36 of the production being consumed compared to terrestrial systems [1]. In response,  
37 phytoplankton have evolved numerous defence mechanisms, varying from morphological and  
38 behavioural to physiological and including the production of various chemical substances [2].  
39 Through the evolution of costly defence mechanisms, grazing may be an important driver of  
40 diversity in phytoplankton communities by allowing coexistence of defence and competition  
41 specialists [3–5]. Coexistence requires that defence benefits comes with a cost, e.g., in the form  
42 of reduced growth [6], but both benefits and costs of defences in phytoplankton have rarely  
43 been convincingly demonstrated [2].

44 Toxic algal blooms are a global phenomenon. Often, the toxins accumulate in the food web  
45 and may negatively affect several trophic levels [7]. Diatoms of the cosmopolitan genus  
46 *Pseudo-nitzschia* can produce the neurotoxin domoic acid (DA) and form dense toxic blooms  
47 with severe consequences to marine organisms and ecosystems, like mass death incidents of  
48 seabirds and marine mammals [7,8]. However, the potential benefits and costs of DA  
49 production have not been established. There is conflicting evidence on whether DA production  
50 is costly; some studies have suggested that cells with increased toxin content have lower  
51 growth rates [9,10], but other experiments have been unable to reproduce these results [11–  
52 13]. Similarly, there is only circumstantial evidence that DA acts as a predator-deterrent. Some  
53 studies have demonstrated reduced predator activity in zooplankton exposed to toxic *Pseudo-*  
54 *nitzschia* [11,14]. Whether this was due to predators becoming intoxicated after consuming  
55 some cells or being exposed to dissolved DA [15], or whether the grazers actively avoid eating  
56 toxic cells is in all cases unclear (a toxic vs a deterrent effect). However, toxic effects are ‘public  
57 goods’ [16] that also benefit non-toxic competitors, and ‘cheaters’ that do not pay the probable  
58 cost of producing the toxin may outcompete the producers. Evolution of such public goods,

59 unless associated with private benefits, is not evolutionary supported. In contrast, the evolution  
60 of toxin production as a ‘private good’, where only the cell producing the toxin benefits from  
61 it, is easy to envisage [16,17].

62 Further evidence of trade-offs and a defensive value of DA is that its production is induced by  
63 chemical cues from copepods, copepodamides [9,18]. Defences are expected to be inducible  
64 only if threats are varying, and the defence is costly [19]. If defences did not have associated  
65 costs, all organisms would evolve towards an equal state of defence [20].

66 Here, we explore the defensive value and trade-offs of DA-production in *P. seriata* exposed to  
67 different concentrations of toxin-inducing copepod cues. We include eight different strains to  
68 account for possible intraspecific variation. We use direct video observations of individual  
69 copepod-prey interactions to quantify the defensive benefits of DA in terms of captured cells  
70 being consumed or rejected, and we quantify the costs of DA-production from changes in cell  
71 division rate. We demonstrate significant benefits and costs of DA-production and thus a clear  
72 trade-off. We further demonstrate threefold differences in growth rate among strains, but that  
73 the trade-off of DA-production is the same. We finally show that DA-production provides both  
74 private and public goods, thus suggesting a potential mechanism for the evolution of apparently  
75 public goods.

## 76 ***Materials and methods***

### 77 **Culturing and species identification**

78 To establish cultures of *Pseudo-nitzschia* for the experiments, live phytoplankton samples were  
79 collected in Øresund, Denmark (55°45’40’’N 12°36’0’’E), in April 2020, by hauling a 20- $\mu$ m  
80 plankton net through the upper (0–5 m) water column. Single cells or chains were micro-  
81 pipetted and placed individually into 96-well plates with L1 medium [21] using a light  
82 microscope (CKX53, Olympus, Tokyo Japan). The cultures were kept at 4 °C at a 16:8 h

83 light:dark cycle and  $120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  using cool white light bulbs. Identification was  
84 performed by transmission electron microscopy (JEOL, JEM 1010, Tokyo, Japan) following  
85 Hasle & Lundholm [22]. All strains included in this study were identified as *P. seriata* (Table  
86 S1, S2).

### 87 **Induction and cost of toxin production**

88 To quantify the costs of toxin production, we performed dose-response experiments with eight  
89 strains of *P. seriata*, where we measured the growth rate as a function of copepodamide  
90 concentration and consequent cellular contents of DA. Cells of different strains were suspended  
91 in L1 medium and added to 200 mL cell-culture flasks. The flasks were exposed to  
92 copepodamides extracted from freeze-dried *Calanus* spp. [23] by coating the inside of the wall  
93 with a mixture of copepodamides dissolved in methanol. The methanol was evaporated using  
94  $\text{N}_2$  gas. Controls underwent the same treatment but received methanol without copepodamides.  
95 Six strains of *P. seriata* were exposed to 0, 10, or 50 pM of copepodamides, while two strains  
96 were exposed to 0 or 50 pM. The flasks were incubated for 72 h at 4 °C or 8 °C (only strain  
97 SKC620) and ca  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  on a 14:10 light:dark cycle. Due to slow release and  
98 quick degradation, the copepodamides coated on the inside of the bottles typically yield an  
99 actual concentration of 1% of the added amount after 12 h, and much less after 72 h [18]. After  
100 72 h incubation, the cells were harvested for enumeration of cell densities and quantification  
101 of cellular DA content. Cell densities were determined by fixing three mL of sample in acidic  
102 Lugol's solution (final concentration 2–3%) and counting a minimum of 400 cells in a  
103 Sedgewick-Rafter chamber using an inverted microscope. Cell growth rates were calculated  
104 using temporal variations in cell densities assuming exponential growth. Average cell size was  
105 determined by measuring 25–30 random cells and cell volume calculated based on Lundholm  
106 et al. [24]. For five of the strains, we also measured dissolved DA. Additional cells from the

107 two strains exposed to only 0 or 50 pM were harvested for use in copepod foraging  
108 experiments.

### 109 **Copepod foraging experiments**

110 We directly observed the feeding response of copepods to the cells by video-recording  
111 individual copepod-cell interactions. The feeding experiments were done in a constant  
112 temperature room (8 °C) in darkness. Adult females of the feeding-current feeding  
113 copepod *Temora longicornis* were glued by their dorsal surface to a human hair [25,26] and  
114 starved overnight in darkness. The other end of the hair was attached to a micromanipulator  
115 and the copepod was submerged in a 10×10×10 cm<sup>3</sup> aquarium with filtered seawater (salinity  
116 27). A different copepod was used for each replicate in each treatment and experiment (three  
117 copepods per treatment). *Pseudo-nitzschia* cells were added to the aquarium and the copepod  
118 response was immediately recorded using a high-speed camera (Phantom V210, Vision  
119 Research, New Jersey, USA). The camera was equipped with lenses to yield a field of view of  
120 about 1.3 x 1.0 mm<sup>2</sup>. A magnetic stirrer gently mixed the water and kept the cells in suspension.

121 We used two of the strains in the feeding experiments (SKC620 and AI420). The copepods  
122 were fed either non-induced control cells or cells that had been exposed to 50 pM of  
123 copepodamides for 72 hours, as described above, to yield different toxin cell quotas. We  
124 conducted three types of experiments: (1) In feeding experiments with strain SKC620, we  
125 recorded four five-minute (0–5 min, 20–25 min, 35–40 min, 55–60 min after adding cells)  
126 sequences at 50 frames per second (fps) at a cell density of 200 cells mL<sup>-1</sup>. (2) In a similar  
127 feeding experiment with strain AI420, we recorded six three-minute (0–3 min, 10–13 min, 20–  
128 23 min, 30–33, 40–43, 50–53) sequences at 100 fps with the same cell density as above. (3) In  
129 a third experiment, we mixed toxic *P. seriata* (strain AI420) with the non-toxic diatom *Ditylum*  
130 *brightwellii*, 100 cells mL<sup>-1</sup> of each species, and recorded sequences (at 100 fps) as above. The

131 two species were easy to distinguish in the videos. The video recordings were analysed to  
132 quantify copepod beating activity and frequency, prey capture, and the fraction of captured  
133 cells that were rejected by the copepods. Additional videos of five strains were recorded using  
134 the same set-up but with 1000 fps to examine potential differences in prey-handling time.

### 135 **Toxin analysis**

136 In all experiments, 45 or 60 mL of each replicate was harvested and divided into 15 mL  
137 centrifuge tubes and centrifuged at  $760 \times g$  for 15 minutes. The supernatants of five strains  
138 (B2N, E9N, D2N, G3N, and H4N) were kept in 15 mL falcon tubes and stored frozen at  $-20$   
139  $^{\circ}\text{C}$  for analysis of dissolved domoic acid. For intracellular toxins, cell pellets were pooled in 2  
140 mL cryotubes and centrifuged again at  $1507 \times g$  for 15 minutes. The pellets were stored frozen  
141 at  $-20$   $^{\circ}\text{C}$ . Toxins were extracted and analysed using liquid chromatography coupled with  
142 tandem mass spectrometry [27].

143 All raw data are available in Olesen et al. [28].

### 144 **Statistical analyses**

145 The effect of the copepodamide treatment on the fraction of time spent beating was analysed  
146 by a mixed-effects logistic regression using the *lme4* R-package [29]. ‘Treatment’ and  
147 ‘Sequence’ were used as fixed effects and ‘Replicate’ as the random effect. Pairwise  
148 comparisons between the different treatments (control – induced, or control – induced – mix)  
149 were done using the Satterthwaite degrees-of-freedom method. The relationship between  
150 growth rate or prey-handling time and cellular DA content was analysed using a linear mixed-  
151 effects model (again using *lme4*) with ‘Strain’ as the random effect. All statistical models were  
152 validated by visual inspection of residual plots.

### 153 **Results**

## 154 **Induction and cost of toxin production**

155 Exposure to copepodamides increased the cellular toxin content in *P. seriata*, but there was a  
156 large variation among strains in terms of the magnitude of the response (Fig. 1). The relative  
157 increase of cellular DA content in the induced treatments compared to the non-induced control  
158 was between 40% and 35 000% (Fig. 1, Table S1). In the five tested strains (B2N, D2N, E9N,  
159 G3N, and H4N), cellular and dissolved domoic acid were not significantly correlated (linear  
160 regression,  $F_{5,9} = 0.6$ ,  $p = 0.72$ ).

161 Growth rates generally decreased significantly with increasing toxin content (Fig. 2, Table S1).  
162 While the intercepts differed significantly among strains, suggesting a threefold variation in  
163 growth rate, the slopes did not differ significantly from one another. Thus, the cost of DA is  
164 the same for all the strains (Fig. 2).

## 165 **Copepod foraging response**

166 *Temora longicornis* copepods use their feeding appendages to create a feeding current from  
167 which prey particles are individually perceived and captured. The copepods react to *Pseudo-*  
168 *nitzschia* cells that are close to or touching the copepod feeding appendages. Depending on cell  
169 orientation, the copepod briefly adjusts the captured particle and brings the cell or chain to the  
170 mouth (Online videos S1–2). Prey handling by the feeding appendages concludes with the cell  
171 either being ingested or rejected (Online videos S1–2). The time spent handling the ingested  
172 particles increased significantly with chain length (Fig. S1) but was independent of cellular DA  
173 content.

174 The two strains used in the foraging experiments both responded to the copepodamides by  
175 increasing their cellular DA content (Fig. 1g, h; Two-way ANOVA,  $F_{3,8} = 29.0$ ,  $p = 0.01$ ), and  
176 a significantly larger fraction of the induced than the control cells caught by the copepods was  
177 rejected (Fig. 3a, b, c). Overall, the fraction of rejected cells increased with the cellular DA

178 content (Fig. 3c). The addition of the alternative prey (*D. brightwellii*) in the mixed experiment  
179 with strain AI420 did not affect the fraction of *P. seriata* cells rejected (Fig. 3a). The *D.*  
180 *brightwellii* cells were rejected at a rate like that of the non-induced *P. seriata* (Fig. 3a).

181 The copepods were initially beating their appendages 100% of the time irrespectively of  
182 treatment (Fig. 4a, c). However, within minutes, exposure to the induced *P. seriata* cells  
183 (AI429) significantly reduced the fraction of time that the copepods spent creating a feeding  
184 current (Fig. 4a, b). Beating time was, however, not different from the controls when the  
185 induced treatment was fed to the copepod in conjunction with *D. brightwellii* (Fig. 4a, b). When  
186 exposed to induced *P. seriata* SKC620, a similar trend was observed (Fig. 4c, d), but the  
187 difference was not statistically significant.

## 188 ***Discussion***

189 Our results show for the first time a convincing enhancement of survival diatoms with  
190 induced toxin production when preyed upon by copepods. The survival strategy comes with  
191 the costs of a significant decrease in growth rate.

### 192 **The defensive benefit of domoic acid**

193 The copepods individually examined captured prey before deciding to ingest or reject it. This  
194 mechanism is similar to that of copepods feeding on toxic dinoflagellates [30], but it is still  
195 unclear how the copepods sense the toxicity of phytoplankton cells without, presumably,  
196 harming them.

197 The increased odds of cell rejection in the induced treatment is a ‘private good’ that only cells  
198 that produce the toxin benefit from [16]. This is similar to the effect of toxins produced by  
199 *Alexandrium* spp. [26,30]. However, in addition, we find that the feeding activity of the  
200 copepods was reduced when offered only induced *P. seriata*. This would be a ‘public good’

201 [16] because the cells that are not producing the costly toxins also benefit. This effect may be  
202 due to a harmful effect of the dissolved DA, which has been found to reduce feeding in krill  
203 [15], or it may be due to some of the toxic cells being eaten and intoxicating the copepod. The  
204 latter may explain why some time elapses before the effect is noticeable [11,25]. Public goods  
205 are in most cases not considered evolutionary stable strategies [16,31]. However, if associated  
206 with a private good, as suggested here, one can envisage a mechanism for their evolution. The  
207 public-good effect is diminished by the presence of alternative prey and would thus only  
208 become 'public' in the later stages of a bloom when toxic cells presumably dominate, and thus  
209 not promote bloom formation [32].

210 The private-good defence is measured as the fraction of captured cells that are rejected by the  
211 copepod. Up to 75% of induced cells were rejected after capture; hence, toxin production may  
212 increase the fitness of the cell provided that the costs are less than the benefits. While the  
213 absolute cost of toxin production is similar among the examined strains, the relative cost is  
214 much larger for slowly growing strains. Thus, in slowly growing SKC620 the relative reduction  
215 in growth rate of induced cells, ~75%, is of the same magnitude as the 75% reduction in  
216 predation risk, while in fast growing strains the benefits outweigh the costs. This suggests that  
217 there are strong trade-offs related to variation in growth rate. Otherwise, fast growing strains  
218 with a very favourable cost-benefit trade-off for toxin production would rapidly outcompete  
219 slowly growing cells with a very limited fitness benefit of toxin production.

220 The presence of an alternative prey may influence copepod prey selection and contribute to the  
221 relative increase of toxic cells [33,34]. However, capture rate (normalized by cell density) of  
222 *P. seriata*, and subsequently the potential grazing mortality, doubled with the addition of *D.*  
223 *brightwellii* compared to when only toxic cells were present as prey because the copepods did  
224 not reduce their beating activity. The addition of the *D. brightwellii* cells did not change the  
225 fraction of induced *P. seriata* cells that were rejected. Thus, responding to predator presence

226 by increasing toxin production is still an adequate response by *P. seriata*, and presumably also  
227 other toxic phytoplankton, in mixed prey suspensions. This is similar to the effect of prey  
228 concentration on the rejection frequency of three species of centric diatoms that thicken their  
229 shells in response to copepod cues [35]. A higher prey concentration increased the odds of  
230 rejection in both defended and undefended cells, but the relative increased odds of rejection in  
231 defended cells remained similar.

232 The inability of previous studies to show evidence of a private-good defence in *Pseudo-*  
233 *nitzschia* spp may stem from the use of wild copepods, or as an artefact of the experimental  
234 design (“black-box” incubations). The present study is the first to use direct observations of  
235 copepod-*P. seriata* interactions [11,12,36]. Predators that are frequently exposed to the  
236 presence of toxic cells may be more tolerant to the toxins [37,38], and the build-up of resistance  
237 is quick, i.e., over a few generations [39]. Thus, collection of wild copepods, e.g., during or  
238 towards the end of a bloom, may influence results. The copepods used in this study were  
239 originally isolated from the same location as the *P. seriata* strains, but have since been kept in  
240 culture for many generations leading, presumably, to a loss of tolerance to DA. However, our  
241 results may still be ecologically relevant. *T. longicornis* generally produce ~6 generations  
242 year<sup>-1</sup> [40,41], in contrast to the longer generation times of *Calanus* spp. used in several earlier  
243 studies [42,43]. Jiang et al. [44] found that *Acartia tonsa* resistance to the toxic dinoflagellate  
244 *Cochlodinium polykrikoides* was relaxed after just two generations of non-exposure. Thus, loss  
245 of tolerance may be fast once toxic cell abundance declines [45,46], and the defence will be  
246 efficient in time for the next blooming event.

### 247 **The cost of toxin production**

248 Defence trade-offs in phytoplankton are notably difficult to establish [2]. However, the fact  
249 that many phytoplankton defences are inducible—they respond to predator-cues by

250 upregulating their defence—suggests that costs should be present [19,47]. We find here a clear  
251 trade-off between predator-induced toxin production and prey growth rate, and the costs were  
252 similar among the tested strains.

253 Several previous experiments have been unable to measure the costs of toxin production in  
254 *Pseudo-nitzschia* [e.g., 10, 27]. Lundholm et al. [9] speculated that this could be due to low  
255 levels of DA that are insufficient to imply measurable differences in growth rates. However,  
256 the trade-off becomes clear in our experiments with the higher levels of DA induced by  
257 copepodamide exposure (Fig. 2). Selander et al. [18] measured the *in situ* concentration of  
258 copepodamides over the course of a year and found values ranging between 40 and 2000 fM.  
259 Thus, the concentrations used in our experiments are within the natural range, and the reduction  
260 in growth rate observed due to increased toxin production is ecologically relevant.

261 The direct metabolic cost of DA-synthesis (see Supplementary information) is low and just 1–  
262 2% of the growth reduction can be accounted for by energy allocated directly to DA-production  
263 (Figure S2). This suggests that other metabolic processes linked to the toxin production are  
264 important. Phytoplankton exposed to predators up-or downregulate thousands of genes, e.g.,  
265 genes related to signal transduction pathways, stress responses, and lipid and nitrogen  
266 metabolism [48,49]. Such associated responses may be energetically costly and may account  
267 for the growth reduction.

## 268 **Toxic blooms and ecosystem implications**

269 The formation of toxic algal blooms have implications to marine ecosystems [7,8]. The ultimate  
270 cause of such blooms is still debated [50], but are frequently attributed to eutrophication. The  
271 ‘private-good’ mechanism demonstrated here improve our comprehension of such toxic  
272 blooms and the factors that allow them to form. A mechanistic understanding of this is key to  
273 predicting their occurrence and how they may be affected by a changing climate.

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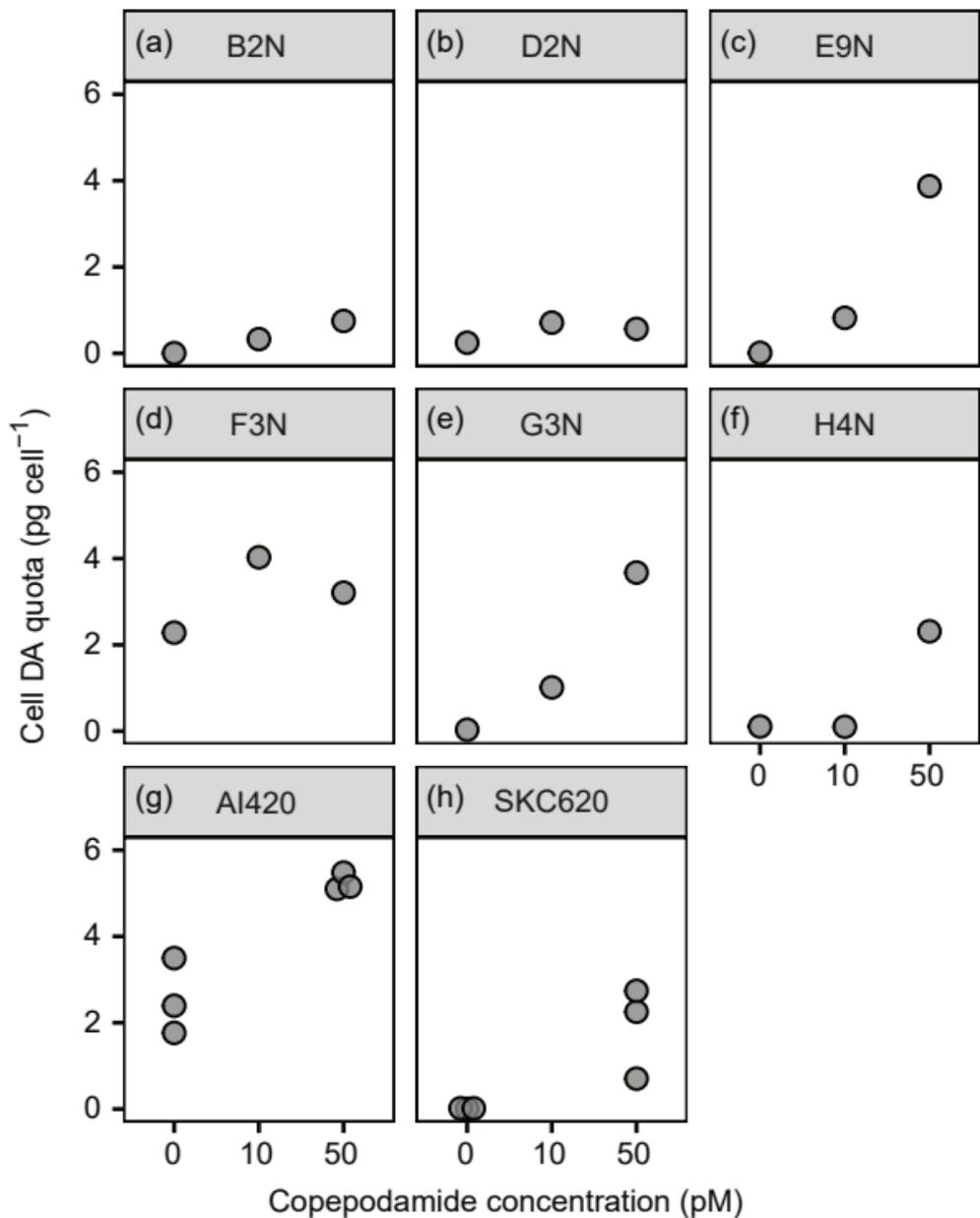
427 **Figure legends**

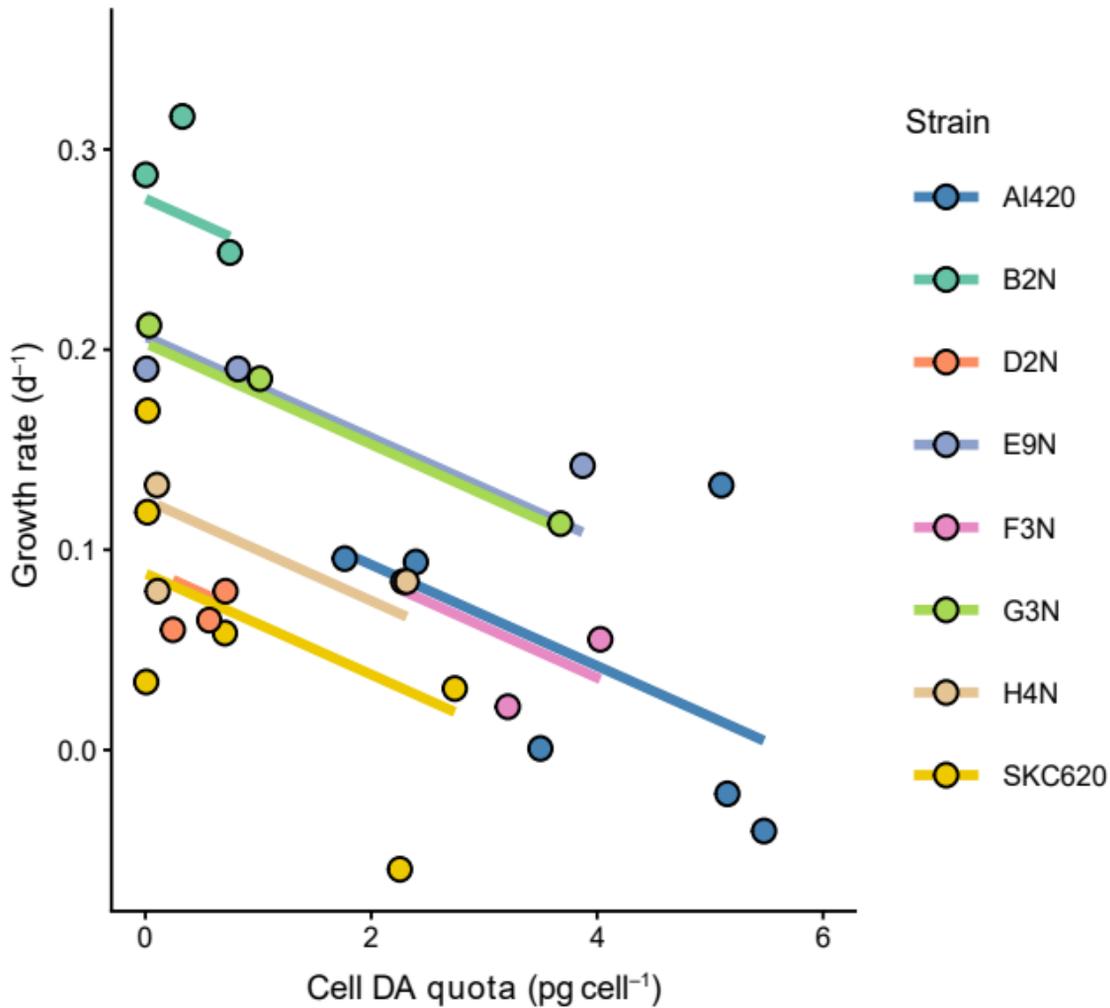
428 Figure 1. Dose-response experiments. Cellular domoic acid (DA) content ( $\text{pg cell}^{-1}$ ) as a  
429 function of the nominal copepodamide concentration for the eight different strains of *P. seriata*.  
430  $n = 1$  for each concentration in a-f, and  $n = 3$  for each concentration in g-h.

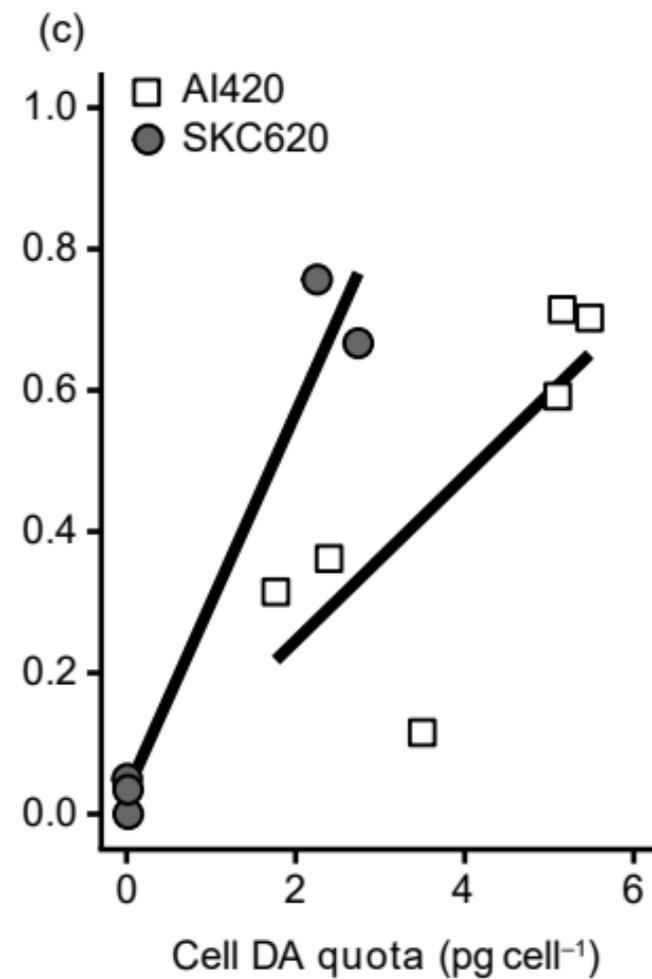
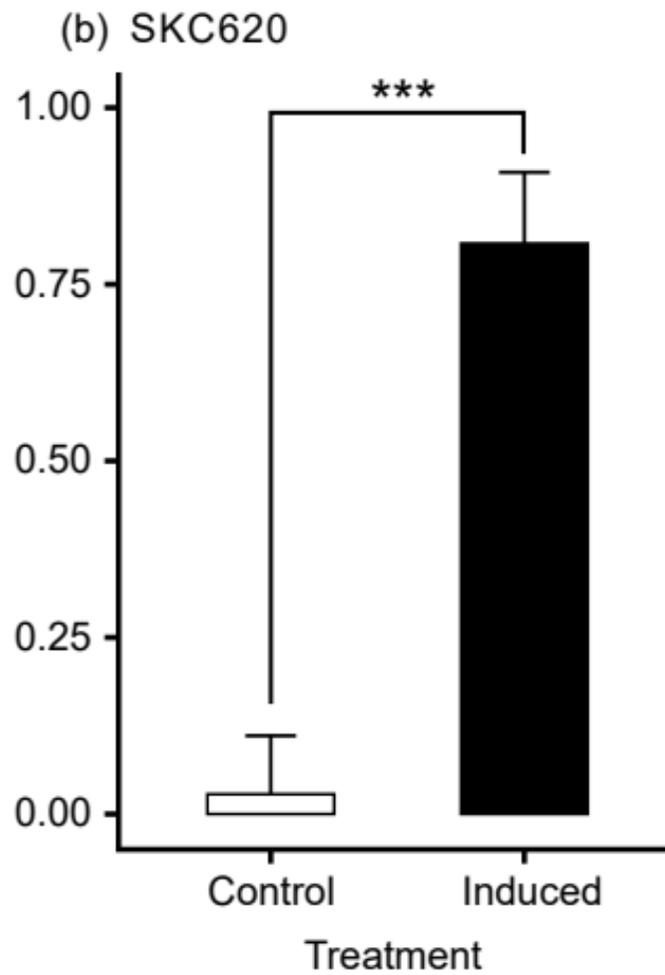
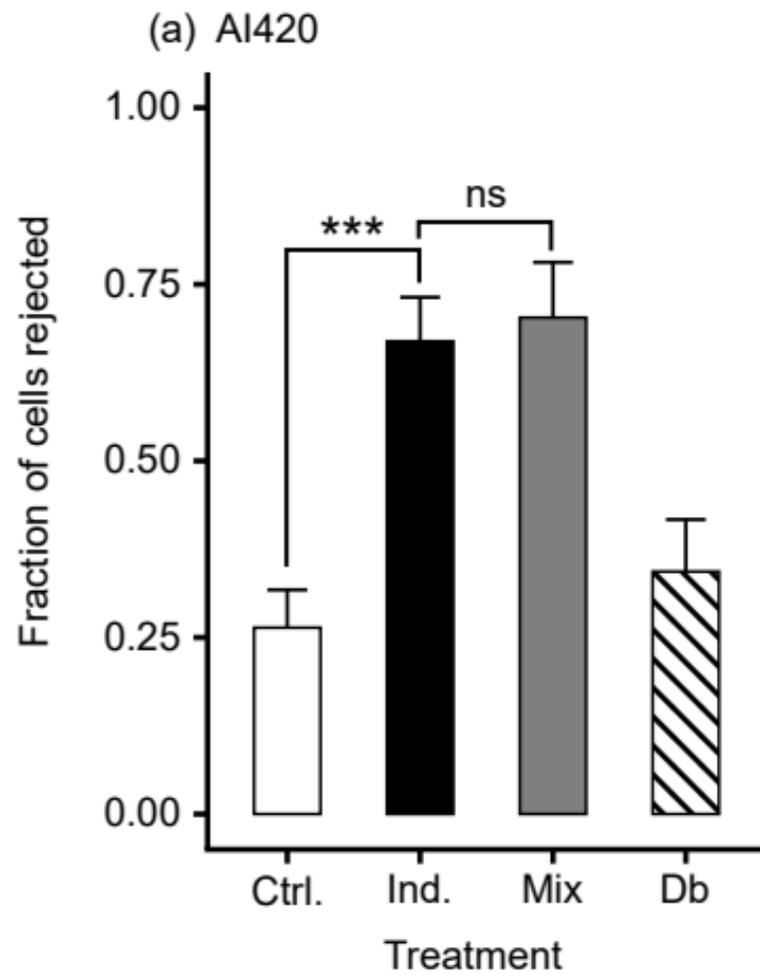
431 Figure 2. Growth-defence trade-off. Growth rate as a function of cellular DA content for all  
432 strains used in this study. A linear mixed-effects model was fit to the data with one slope and  
433 random intercepts. The estimated slope (with 95% confidence intervals) is  $-0.025 \text{ d}^{-1} (\text{pg DA}$   
434  $\text{cell}^{-1})^{-1} [-0.032, -0.018]$  ( $F_{1,25.3} = 13.2, p < 0.001$ ).

435 Figure 3. Defence efficiency. The fraction of cells rejected in strain (a) AI420 and (b) SKC620.  
436 Mix: the fraction of induced *P. seriata* cells rejected in the mixed experiment. Db: fraction of  
437 *D. brightwellii* cells rejected in the mixed experiment. (c) Relationship between the fraction of  
438 rejected cells and the cellular DA content. Values in (a) and (b) are the fractions from all  
439 captures across several copepods recorded and error bars show 95% Wilson Score Interval ( $n$   
440  $= 48-287$ ). In (c) they are the fractions for each copepod and include the control and induced  
441 treatments. One data point was removed in (c) due to the copepod only capturing two cells  
442 (both were rejected). Asterisks indicate significant difference between treatments, \*\*:  $p < 0.01$ ,  
443 \*\*\*:  $p < 0.001$ . ns: not significant. Odds ratios (with 95% confidence intervals) when  
444 comparing 'Control' and 'Induced' treatments are (AI420) 5.71 [3.86, 8.54] and (SKC620)  
445 83.99 [21.76, 563.50].

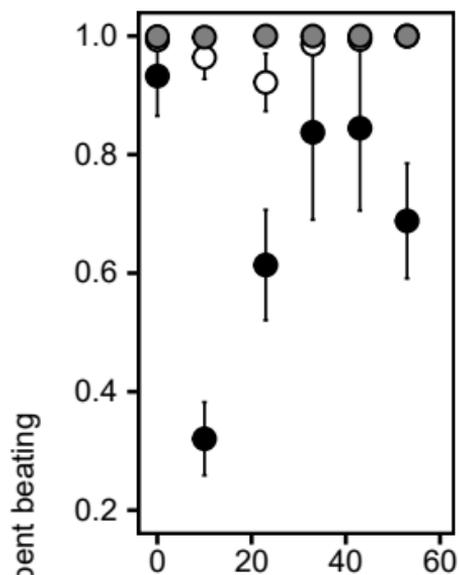
446 Figure 4. Copepod beating frequency. Fraction of time the copepods spent beating starting as  
447 the cells are added (a, c) and averaged for each treatment (b, d) in the foraging experiments  
448 with strains AI420 (top row) and SKC620 (bottom row). Lower case letters refer to significant  
449 differences between treatments according to pairwise comparisons ( $p < 0.05$ ). Colours referring  
450 to treatments in the bar charts (b, d) apply also in (a) and (c). Error bars in all panels show  
451 standard error ( $n = 3$ , except in 'Mix' where  $n = 2$ ).



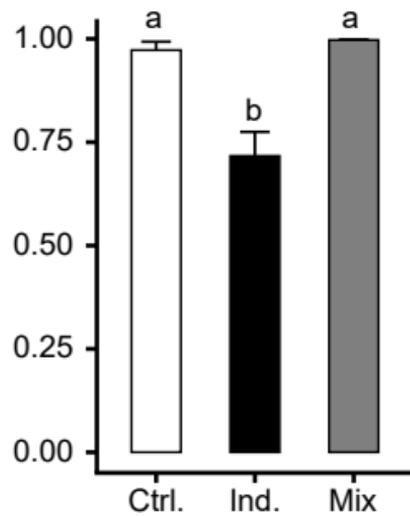




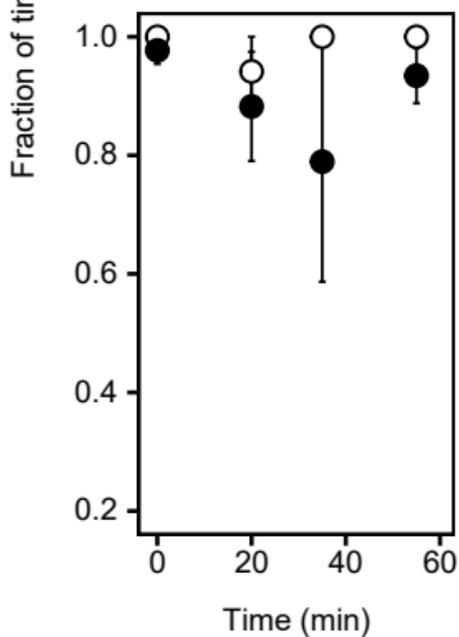
(a) AI420



(b)



(c) SKC620



(d)

