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# REVERSIBLE COLONY FORMATION AND THE ASSOCIATED COSTS IN *SCENEDESMUS OBLIQUUS*

Authors:

Dania Albini*, Mike S. Fowler, Carole Llewellyn, Kam W. Tang

Department of Biosciences, Swansea University, Swansea, SA2 8PP, United Kingdom

*Corresponding author: [Dania Albini: dania.albini@swansea.ac.uk]

Address: Department of Biosciences, Swansea University, Swansea, SA2 8PP, United Kingdom

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**ABSTRACT:**

Grazer-induced colony formation as a defence strategy in microalgae such as *Scenedesmus* species has been widely reported, but the associated costs and reversibility of the colonies are rarely studied. We experimentally showed that *S. obliquus* formed chained colonies in the presence of a predator, including predators separated from the algae by a membrane, but quickly reverted to single cells after the removal of the predator – a defining characteristic of an inducible defence. We detected the stress indicator astaxanthin esters in the algal populations in the presence of grazers, but not when grazers were absent. We found significant costs associated with *S. obliquus* colony formation in terms of lower population growth rate, lower photosystem II efficiency and lower cellular Chlorophyll *a* content. These results together show that colony formation as an inducible defence in *S. obliquus* against grazers comes at a substantial cost such that the defence must be switched off and the colonies revert to single cells when the predation risk disappears.

29 **INTRODUCTION**

30

31 In aquatic environments, morphological anti-predator traits are an important defence for
32 microalgae to reduce predation risk, e.g., colony formation (Trainor, 1991; Lürling and Beekman,
33 1999; Jakobsen and Tang, 2002). The green microalga *Scenedesmus* sp., for instance, is usually
34 present as single cells, but it can form chained colonies in the presence of grazers (Tollrian and
35 Harvell, 1999). Colonies are the result of cell divisions without separation, the process of which is
36 limited by cell multiplication and growth (Pickett-Heaps and Staehelin, 1975; Trainor et al., 1976).
37 As most grazers are size selective, this increased-size defence reaction reduces the predation risk for
38 the alga (Hessen and van Donk, 1993; Lürling and van Donk, 1996). Physical contact with the
39 grazers is not required to elicit the response, as the predation risk can be communicated via
40 chemical cues from the grazers (Hessen and Van Donk, 1993; Lampert et al., 1994; Lürling, 2000).
41 By definition, inducible defensive phenotypes should revert to the original phenotypes once
42 the predation risk has disappeared (Tollrian and Harvell, 1999; Van Donk et al., 2011). Defensive
43 colony formation by *Scenedesmus* spp. has been widely studied, but reversibility from the colonial
44 to the unicellular form has rarely been tested (Verschoor, et al. 2009).
45 According to defence theory, the algae should pay some costs for their predator-induced
46 defence response (Mole, 1994; Agrawal, 1998); otherwise the defensive (colonial) form would be
47 the norm (Dodson, 1989). However, while the benefit of defensive traits for prey survival is clear,
48 the costs are often unknown *a priori* and can be difficult to identify (Lürling and Van Donk, 2000).
49 Thus far, evidence has suggested higher settling velocities for *Scenedesmus* colonies, thereby
50 removing the algae from the euphotic zone (Lampert et al., 1994; Lürling and Van Donk, 2000).
51 Other possible costs can be considered for *Scenedesmus* colony formation as well: 1) reduced
52 nutrient and light uptake due to the "package effect" (Kirk, 1994), resulting in lower growth rate; 2)
53 decreased photosystem II (PSII) efficiency (Lürling and Van Donk, 2000); and 3) decreased
54 Chlorophyll *a* content (Lürling and Van Donk, 2000; Yang et al., 2009).
55 Many green algae are known to accumulate secondary keto-carotenoids such as Astaxanthin
56 (Ax) and its derivatives Astaxanthin-esters (Ax-E) as part of the xanthophyll cycle when exposed to
57 stress such as high irradiance or UV (Figure 1). These stresses lead to the formation of reactive
58 oxygen species (ROS), and Ax and Ax-E as antioxidants can protect the cells from ROS damages
59 (Lemoine and Schoefs, 2010). As such, Ax and Ax-E accumulations have been used to indicate
60 photo-oxidative stress in algae (Quin et al., 2008; Aburai et al., 2015), but there is no prior report
61 that links their accumulation to predation-related stress.

62

63 In this study, we tested for colony formation in *Scenedesmus obliquus* induced by chemical
64 cues from a grazer with different levels of feeding activity, and the associated costs in terms of
65 population growth rate, photosystem II efficiency and cellular Chlorophyll *a* content. We
66 hypothesised that direct or indirect grazing cues would induce colony formation, but non-feeding
67 grazers would induce fewer colonies than actively feeding grazers. Furthermore, we tested the
68 alga's ability to revert to unicells and to recover the costs following the disappearance of the
69 grazers. Lastly, we investigated, for the first time, the production of Astaxanthin (esters) by *S.*
70 *obliquus* in response to grazing.
71
72
73 **METHODS**
74
75 To investigate how grazers affected colony formation and reversal in microalgae, and assess
76 potential costs to the algae, we carried out a series of laboratory experiments.
77 ***Organisms:***
78 The green alga *Scenedesmus obliquus* (Turpin) (recently renamed *Tetradesmus obliquus*
79 (Turpin) Wynne (2016)) was bought from the Culture Collection of Algae and Protozoa (CCAP,
80 strain number 276/6A), and grown in BG-11 medium (Sigma-Aldrich 73816 FLUKA) in batch
81 culture in 250-mL flasks. *S. obliquus* is commonly found as single cells; however, it can form
82 colonies in the presence of grazers (Lürling and van Donk, 2000; Zhu et al., 2015). The freshwater
83 zooplankton *Daphnia magna* Straus, 1820 (Cladocera) was obtained from the Leibniz-Institute of
84 Freshwater Ecology and Inland Fisheries (Germany). Genetically identical individuals originated
85 from a single female were fed daily *ad libitum* with a mixture of two algae: *S. obliquus* and
86 *Raphidocelis subcapitata* (Sphaeropleales), and kept in glass beakers with spring water (Evian
87 mineral water: pH = 7.2; $Ca^{2+}$ = 78 mg $L^{-1}$). *R. subcapitata* (CCAP, strain number 278/4) was
88 cultivated in the same conditions as *S. obliquus*. Algae and zooplankton cultures were grown at a
89 temperature of 21 ± 2°C and a light intensity of 80-90 µmol photons $\cdot m^{-2} \cdot s^{-1}$ in an 18 hours light: 6
90 hours dark cycle.
91 Dialysis bags are semi-permeable membranes used in separation techniques for the removal
92 or exchange of molecules based on different pore sizes. The dialysis bags used in this study
93 (Medicell Membranes Ltd, London) and had a pore size of 12-14 kD. The bags allowed the passage
94 of *Daphnia* infochemicals but prevented physical contact between *D. magna* and *S. obliquus*. The
95 bags were washed following the manufacturer's instructions before use.
96
97 ***Colony induction experiment:***

98 The experimental set-up consisted of dialysis bags placed in 500 mL glass beakers (Figure 2),
99 divided as follows: three replicates of control beakers each of: 1) Single-celled *S. obliquus* inside
100 (C1B) and outside (C1O) the dialysis bag to determine the background level of colony formation in
101 the absence of grazers. The treatment beakers consisted of three replicates each of: 2) single-celled
102 *S. obliquus* + nutrients inside the dialysis bag (T1B) and mineral water + nutrients + *D. magna*
103 outside the dialysis bag (T1O) to assess colony formation induced by non-feeding *D. magna*; 3)
104 single-celled *S. obliquus* + nutrients inside the dialysis bag (T2B) and single-celled *S. obliquus* with
105 *D. magna* + nutrients outside the dialysis bag (T2O) to assess colony formation induced by actively
106 grazing *D. magna*.

107 Before the start of the experiment, *S. obliquus* population was grown at a temperature of 21 ±
108 2°C under a cold light of 80-90 μmol photons$\cdot$ $m^{-2}\cdot s^{-1}$ and a photoperiod of 18 hours light: 6 hours
109 dark. BG-11 medium was used as source of nutrients. An initial concentration of ca. $5 \times 10^5$ cells
110 $mL^{-1}$ of unicellular *S. obliquus* in exponential phase was added to the dialysis bags and relevant
111 beakers. The total volume of the dialysis bag was 70 mL; each bag was sealed at both ends with
112 clips. 10 genetically identical *D. magna* adults (4 ± 2 days old) were used in each predator treatment
113 beaker. All the beakers were placed in front of a cold light of 80-90 μmol photons$\cdot$ $m^{-2}\cdot s^{-1}$ at a
114 temperature of 21 ± 2°C and in a photoperiod of 18 hours light: 6 hours dark.
115 The beakers were manually shaken and their positions changed daily to ensure uniform
116 exposure to light throughout the experiment. Moreover, the dialysis bags were gently shaken and
117 inverted in the beaker twice a day to avoid sedimentation of algae and to mix the medium. The
118 experiment lasted for 3 days. On Day 0 and Day 3, aliquots were collected for the following
119 analyses: pigments, quantum yield (PSII efficiency) and cell (colony) counts. Additional samples
120 for cell and colony counts were taken on Day 1.
121
122 ***Colony reversibility experiment:***
123 A second experiment was conducted to test whether *S. obliquus* colonies were able to revert
124 to single cells once the grazing risk had disappeared. For this experiment we used *S. obliquus*
125 populations recovered from the *Colony induction experiment*.
126 At the end of the *Colony induction experiment*, aliquots were transferred from the different
127 beakers into new beakers filled with 70 mL of deionized water and BG-11 nutrient medium, to
128 create an initial inoculum of ca. $3 \times 10^5$ cells $mL^{-1}$ of algal population dominated by single cells or
129 colonies (Figure 3). The new beakers were exposed to the same light and photoperiod conditions as
130 before, and were manually shaken and their positions changed daily to ensure uniform exposure to
131 light throughout the experiment. The experiment lasted for 3 days. On Day 0 and Day 3, aliquots

132 were collected for the following analyses: pigments, quantum yield (for PSII efficiency) and cell
133 (colony) counts. Additional samples for cell (colony) counts were taken on Day 1.
134
135 ***Measurement of pigments***

136 The pigment contents of the algal populations were determined on Day 0 and Day 3, using
137 the method described by Zapata et al. (2000) with HPLC (High Performance Liquid
138 Chromatography). A 20-mL aliquot was transferred from each algal population with a sterile
139 syringe into an Eppendorf tube and pellets were created from these aliquots after centrifugation.
140 The samples were extracted with 90% HPLC grade acetone (Sigma-Aldrich) and sonicated with a
141 sonicator probe (Fisher Scientific) for 1 minute at 40 Hz to lyse the cells. Once the pigments were
142 extracted, each pellet was centrifuged at 6000 g for 5 minutes and 50 µL of the supernatants
143 containing pigments were used for HPLC analysis. The HPLC system had a 150 × 4.6 mm column
144 (Waters spherisorb ODS2, particle size diameter of 5 µm) with a flow rate of 1 mL $min^{-1}$ and the
145 solvent gradient described in Zapata et al. (2000). Astaxanthin, its ester forms and other pigments
146 (not reported) were identified by comparing our retention times and diode array spectra with those
147 reported by Jeffrey et al. (1997). Specific pigment contents were expressed as percentages of peak
148 area relative to the total peak area of all the pigments. Chlorophyll *a* was quantified against
149 reference standard (Sigma-Aldrich, 96145).

150

151 ***Quantum yield measurement (QY)***
152 PSII efficiency was measured in samples collected at the same time of day during the light
153 period (ca. 80-90 µmol photons $m^{-2}$ $s^{-1}$) as effective quantum yield (QY) using an Aquapen (AP-C
154 100, Photon Systems Instruments). Fluorescence nomenclature and calculation was done according
155 to the manufacturer's instructions. At the end of the light period, a 2-mL aliquot was transferred
156 from each algal population into a cuvette which was then inserted into the Aquapen. The variable
157 fluorescent ($F_v$) and the maximal fluorescence intensity ($F_m$) were measured with an excitation
158 wavelength of 455 nm and emission wavelengths of 667-750 nm. The mean of three measurements
159 was used to calculate the effective efficiency of PSII $e^-$ -flow as $F_v/F_m$. Afterward, the aliquot was
160 recovered for cell counts (see below).
161
162 ***Cell (colony) counts and measurement of algal growth rate***
163 Aliquots recovered from QY measurements were fixed with Lugol's solution (Sigma-
164 Aldrich 62650-1L-F) and stored in a dark refrigerator (~5 °C). Samples were counted within 15

165 days using a haemocytometer under a Leica inverted microscope (400× magnification). Numbers of
166 single cells, number of colonies and number of cells per colony were recorded. The specific growth
167 rates (μ) were calculated for the periods of Day 0–Day 1 and Day 1–Day 3 using the equation: $\mu =$
168 $[\ln (X_2 / X_1)]/(t_2 - t_1)$, where $X_1$ and $X_2$ are the counts of total cells at time $t_1$ and $t_2$, respectively.
169

170 ***Statistical analysis***

171 Statistical analyses were performed using R studio software (v. 1.1.383). One-way ANOVA
172 was used to test for differences between treatments in terms of (*i*) the change in astaxanthin %, QY
173 values and pigment percentages; (*ii*) the number of cells in colonies; recorded over the course of the
174 experiments. Differences between QY were calculated using the mean QY value of three replicates
175 of each treatment on Day 0 and Day 3. These responses were considered during both colony
176 formation and colony reversal. Model residuals were tested with a Shapiro-Wilks test, and no
177 evidence was found suggesting deviation of residuals from a Normal distribution ($p > 0.05$ in all
178 cases). Levene's test showed no deviation from the assumption of homogeneity of variance across
179 groups ($p > 0.05$ in all cases). Post-hoc Tukey tests tested for differences between colonies induced
180 by non-feeding and actively feeding *Daphnia*. The level of statistical significance was set at $\alpha =$
181 $0.05$.
182
183

184 **RESULTS**

185 ***<u>Colony induction experiment:</u>***

186 Astaxanthin esters were absent in all the treatments and controls on Day 0, but they appeared
187 on Day 3 in the algal populations treated with *D. magna*, while they remained absent in the controls
188 (Figure 4A). Differences in QY values across treatments were detected ($F_{4,10} = 284.5$, $p < 0.001$). *S.*
189 *obliquus* populations exposed to *D. magna* showed a drop in QY; on the contrary, *Daphnia*-free
190 populations of *S. obliquus* showed an increase in QY over time (Figure 4B).

191 All the replicates (controls and treatments) had the same initial concentration of Chlorophyll *a*
192 per cell (mean 0.139 ng $cell^{-1}$ ± 0.005 s.e.). On Day 3, cellular Chlorophyll *a* content decreased
193 drastically in all algal populations under the direct or indirect influence of *D. magna* (mean change:
194 -0.13 ng $cell^{-1}$ ± 0.007 s.e.), whereas in the Controls the cellular Chlorophyll *a* content increased
195 significantly (mean change: 0.102 ng $cell^{-1}$ ± 0.001 s.e.; Figure 4C).
196

197 There was no evidence of any difference between direct contact with *D. magna* (T2O) and
198 exposure to *D. magna*'s chemical cues (T1B, T2B) in the formation of *S. obliquus* colonies ($F_{2,6} =$

199 0.273, $p = 0.77$; Figure 5). Controls without *D. magna* had a low and stable amount of multi-celled
200 clusters throughout the experiment. The number of cells per colony increased over time in the
201 treatments exposed to *D. magna* stimuli, with an increase in 8-celled colonies by Day 3 in all
202 replicates (Fig. 5, $F_{4,10} = 286.6$, $p < 0.001$).

203 As expected, the algal populations in direct contact with *D. magna* decreased because they
204 were being grazed. However, even the populations inside the dialysis bags showed lower growth
205 rates ($\mu$) in the presence of *D. magna* chemical cues: in the first period (Day 0–Day 1) algal
206 population growth rates in the presence of *D. magna* chemical cues ($\mu = 0.02$ and $0.01$ $d^{-1} \pm 0.004$
207 s.e. for T1B and T2B, respectively) were much lower than that of the control populations
208 ($\mu = 0.69$ $d^{-1} \pm 0.000$ s.e for both C1B and C1O). In the second period (Day 1–Day 3), $\mu$ was still
209 lower in algal populations exposed to *D. magna* chemical cues ($\mu = 0.03$ and $0.04$ $d^{-1} \pm 0.004$ s.e.
210 for T1B and T2B, respectively) than in the control populations ($\mu = 1.13$ and $1.07$ $d^{-1} \pm 0.02$ s.e. for
211 C1B and C1O, respectively).

212

213

214 ***Colony reversibility experiment:***

215 Astaxanthin esters were present in all of the starting populations that were previously exposed
216 to *D. magna*, and these pigments were no longer present on Day 3 following removal of the grazers
217 (mean decrease: -5% ± 0.73 s.e., Fig. 6a). Astaxanthin esters were always absent in the control
218 populations that were never exposed to *D. magna* (C1B in Fig. 3).

219 QY values increased between Day 0 and Day 3 in all populations with or without pre-
220 exposure to *D. magna* ($F_{3,8} = 10.97$, $p < 0.005$, Figure 6B). The Chlorophyll *a* level was low and
221 similar for both control and pre-exposed populations on Day 0. At the end of the experiment, the
222 amount of Chlorophyll *a* per cell increased in the control (from 0.3 to 0.8 ng $cell^{-1}$) but less so in the
223 pre-exposed populations (ca. +0.05 ng $cell^{-1}$; Fig. 6C).

224

225 There was a significant decline in the number of cells per colony from Day 0 to Day 3 in the
226 pre-exposed populations during the reversibility experiment, with the 8-celled colonies disappearing
227 by Day 3 (Fig. 7, $F_{3,8} = 8.655$, $p = 0.006$). The control population maintained a low and stable
228 number of small colonies.

229 In the first day after removal from the grazer, the algal populations had a low growth rate
230 ($\mu = 0.03$, $0.03$ and $0.07$ $d^{-1}$, for T2O, T1B and T2B respectively). As the experiment progressed

231 (Day 1–Day 3), the growth rates of the pre-exposed populations increased and became comparable
232 to that of the control population: $\mu$ = 1.40 $d^{-1}$ for C1B and T1B, 1.37 $d^{-1}$ for T2B and 1.39 $d^{-1}$ for
233 T2O.
234

235 **DISCUSSION**

236

237 The ability to defend against predators is a major evolutionary driving force in organisms' life
238 histories; this is particularly the case for microalgae that lack mobility or physical refuge (Van
239 Donk et al., 2011). *Scenedesmus* is a cosmopolitan freshwater algal genus with more than 1300
240 known species distributed globally (Coesel and Krienitz, 2008). Due to its ability to form easily
241 recognisable chained colonies in response to grazing, it is widely used as a model organism in the
242 study of morphological defence (e.g. Lürling and Van Donk, 1997; Lürling, 1999). While many
243 reports describe *Scenedesmus* colony formation as an inducible defence response, test of
244 reversibility of the colonies is rare in the earlier studies. Likewise, there is limited evidence of the
245 costs associated with colony formation.
246 In our experiments, unicellular *S. obliquus* formed chained colonies in the presence of *D.*
247 *magna* even without physical contact with the grazer, suggesting that the predation risk could be
248 communicated via chemical cues. Moreover, the number of cells per colony increased over time,
249 from 2-celled colonies on Day 0 to 8-celled colonies on Day 3 (Figure 5). These observations are
250 consistent with other studies of defensive colony formation in microalgae against grazers (Lampert
251 et al., 1994; Wiltshire and Lampert, 1999; Tang 2003).
252 Unlike terrestrial ecosystems where grazing is typically non-lethal to the plants, grazing in
253 plankton often means death to the algal cells. *Daphnia* species are among the most dominant
254 grazers in freshwater systems (Sterner, 1989), able to exert strong top-down control on algae
255 leading to a 'clear water phase' in many lakes (Deneke, 1999). From the perspective of *S. obliquus*,
256 it may be advantageous (or even necessary) for the algae to react to the mere presence of the grazer
257 before grazing occurs. In contrast to previous work (e.g. Lampert et al., 1994) and our expectation,
258 the extent of colony formation (in terms of the increase in number of colonial cells $mL^{-1}$ over time)
259 was almost identical between the algal populations exposed to non-feeding *D. magna* and those
260 exposed to actively grazing *D. magna*. In the earlier study (Lampert et al., 1994), the grazer was
261 starved for 48 hours prior to the experiments, whereas in our study *D. magna* was starved only
262 during the experiments. Our observations therefore suggest that the release of chemical cues does
263 not depend on continuous, active feeding; rather, chemical cues resulting from recently fed grazers
264 were sufficient to trigger colony formation.

265

266 The benefit of inducible defences is, by default, enhanced survival of the organism, but the
267 associated cost(s) can be difficult to identify, and as a consequence there is very limited information
268 on costs in the literature. Formation of colonies requires the production of special cellular structures
269 and materials (Pickett-Heaps and Staehelin, 1975; Trainor, 1998), which may divert resources from
270 other vital cellular functions. The enlarged volume-to-surface ratio of the colonies may also
271 decrease the alga's resource acquisition ability (Kirk, 1994). We showed that both PSII efficiency
272 and cellular Chlorophyll *a* content decreased significantly during *S. obliquus* colony formation,
273 either of which could lead to a reduced growth rate, as we also confirmed in our experiments. The
274 observation that Astaxanthin esters increased in *S. obliquus* when the cells were exposed to grazer is
275 also interesting. Astaxanthin esters are known to protect algal cells from photo-oxidative stress
276 (Lemoine and Schoefs, 2010), but it is unclear what protective benefits they served against grazing.
277 We may speculate that the chemical cues released by the grazer may have contained oxidative
278 substances; as such, the pigments may have been a response to this grazer-associated oxidative
279 stress rather than to grazing stress per se. Regardless, our observations suggest that Astaxanthin
280 esters may be used to indicate a wider range of stress than previously known.

281 Interestingly, our results differ from Lürling and Van Donk (2000), who did not observe any
282 change in QY in grazer-influenced cells. It is useful to point out that Lürling and Van Donk (2000)
283 dark-adapted their samples and their measurements represented the maximum QY. In our study, we
284 chose to measure effective QY without dark-adaptation, which was more indicative of the real-time
285 photosynthetic activity of the cells (Murchie and Lawson, 2013). The omission of dark adaptation
286 also minimised the possibility of the cells 'recovering' while away from the grazer's influence (cf.
287 Lürling and Van Donk, 2000). Moreover, effective QY is considered a good physiological indicator
288 of how photosynthetic organisms respond to environmental stress (Rascher et al., 2000). Deviations
289 of effective QY from the control usually indicate a reversible down-regulation of PSII
290 photochemistry rather than irreversible damage to the photosynthetic apparatus (e.g. Demmig-
291 Adams et al., 1996). This is in agreement with our second experiment where we saw rapid recovery
292 of the effective QY values during colony reversal (Figure 6B). In our experiments we observed a
293 lower growth rate than in the previous report; this difference may be partly attributed to the fact that
294 we conducted our experiments under 18:6 light-dark cycle, rather than continuous light (cf. Lürling
295 & van Donk, 2000).

296 Faced with the high costs associated with colony formation, *S. obliquus* reverted to unicells
297 upon removal from the grazer, a prerequisite trait for colony formation to be described as an
298 'inducible' defence (Tollrian and Harvell, 1999). As expected, Astaxanthin esters also disappeared
299 completely once the grazer was removed. Interestingly, but perhaps not surprisingly, colony

300 formation and reversal occurred at different rates: in the *Colony induction experiment*, unicells
301 changed to colonial form at a rate of $71 \times 10^3$ cells $mL^{-1}$ $d^{-1}$. This was considerably higher than the
302 rate at which cells in colonial form changed back to unicells (*Reversibility experiment*: $28 \times 10^3$
303 cells $mL^{-1}$ $d^{-1}$). Colony formation protected the cells from certain death (grazing) whereas the
304 associated costs, albeit substantial, were not necessarily fatal to the cells. It is therefore reasonable
305 to argue that colony formation by *S. obliquus* under predation threat carried a much higher urgency
306 than the reverse process. At the end of the reversibility experiment (i.e., after 3 days without
307 predator cues), there were still about 5% cells in colonial form in the pre-exposed populations,
308 compared to only 0.3% in the control populations. Consistent with this observation, the costs were
309 also not fully recovered for the pre-exposed algal populations: While their growth rate and the PSII
310 efficiency recovered to being comparable to the control populations, their cellular Chlorophyll *a*
311 content still lagged behind that of the control populations. It therefore appears that the process of
312 Chlorophyll *a* synthesis may require a longer time to return to normal.
313
314
315 **CONCLUSIONS**
316 The algal genus *Scenedesmus* is a very useful model organism to study the ecology and
317 evolution of morphological defences against predators, but thus far the literature lacks detailed
318 information on the associated costs and colony reversibility. Here we not only showed that colony
319 formation by *S. obliquus* was reversible upon removal of the grazing threat; we also quantified the
320 costs associated with colony formation. This information will be useful for further cost-benefit
321 analysis of this defensive trait, especially when in combination of other environmental constraints.
322 The discovery of the production of Astaxanthin-esters, a commercially valuable antioxidant, under
323 predation-related stress is also interesting and deserves further investigation.
324
325
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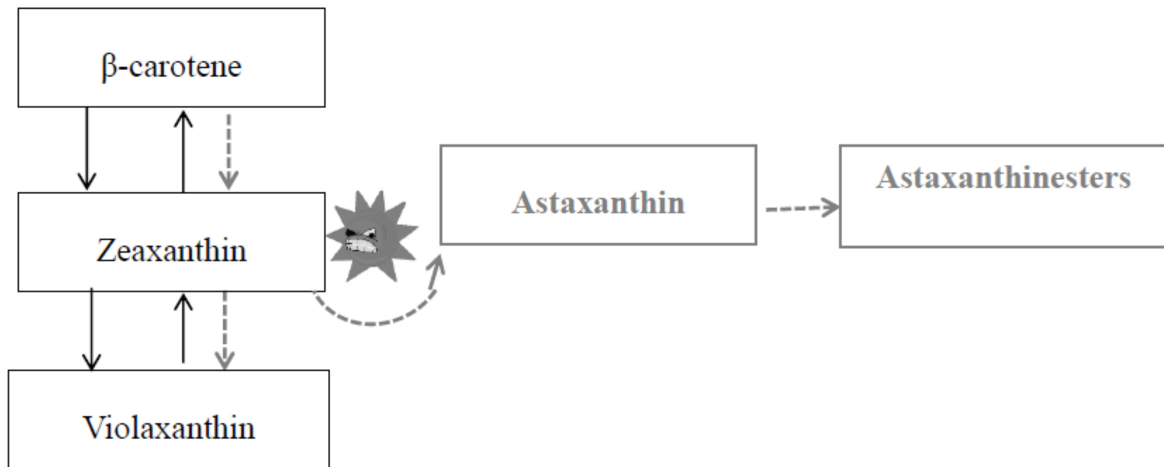
414

415 **Figures:**




416

417 Figure 1. Simplified schematic of a synthesis pathway of the pigments Astaxanthin and
418 Astaxanthin-esters in *Scenedesmus* sp. (Lemoine and Schoefs, 2010). In the absence of stress,
419 Zeaxanthin is disaggregated from energy dissipation and converted back to Violaxanthin (black
420 line). When the cell is exposed to stress, Violaxanthin is transformed to Zeaxanthin, which is then
421 converted to Astaxanthin and Astaxanthin-esters (grey dotted line).

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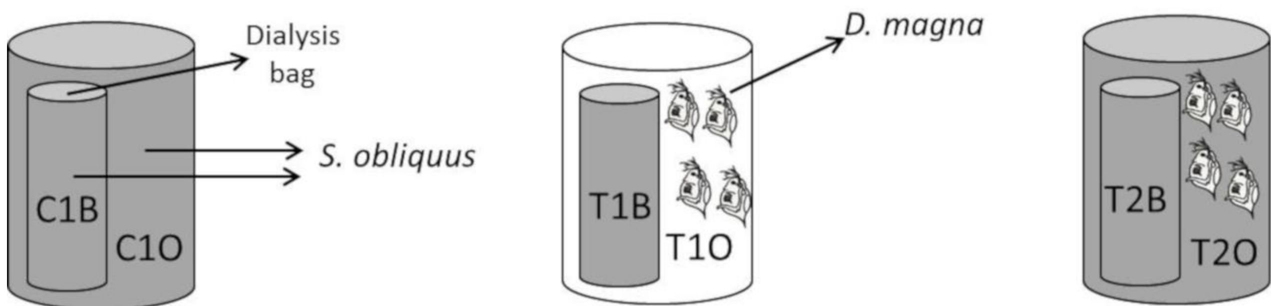


424

425 Figure 2. Schematic of the experimental design for colony induction. Each beaker contained a
426 dialysis bag with *Scenedesmus obliquus* single cells (grey shading). Treatment beakers also
427 contained *Daphnia magna* (*Daphnia* figure) outside the dialysis bag, with (grey) or without (white)

428 *S. obliquus* as food. All beakers contained Evian water with nutrients (BG-11). T = treatment, C =
429 control, B=bag, O= outside, 1 and 2 are to distinguish the first and second treatments respectively.
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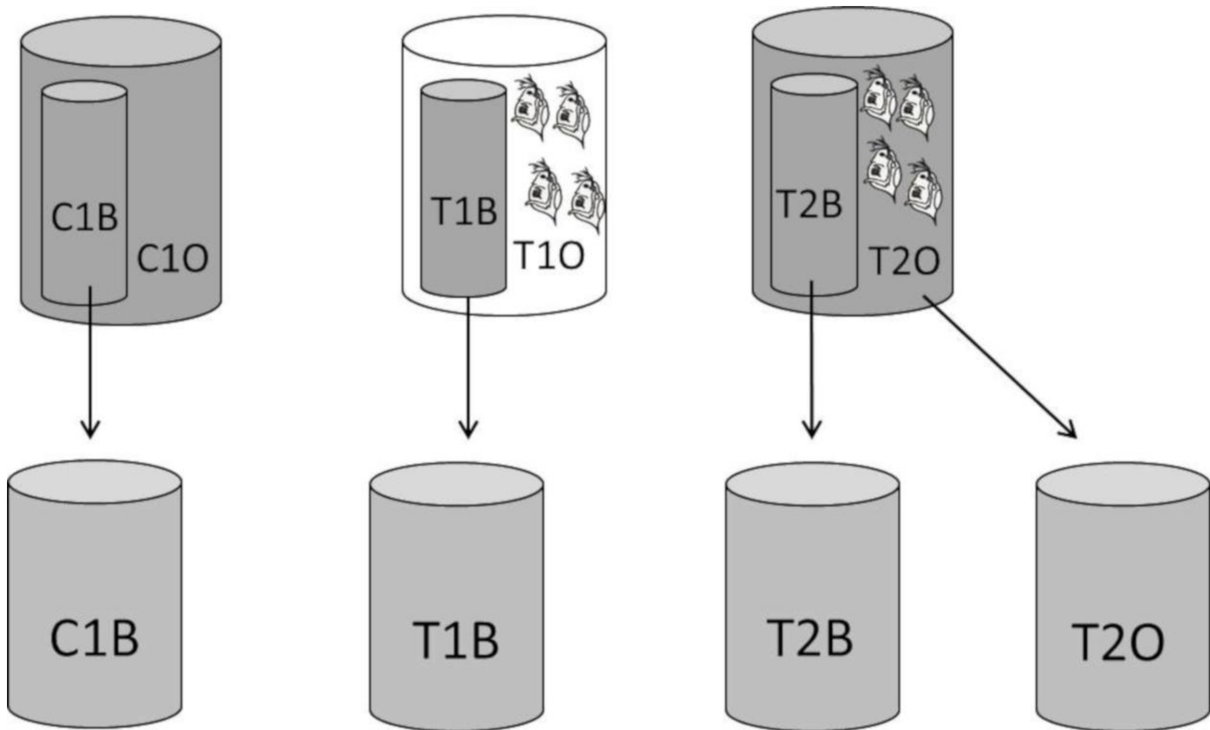

431

432 Figure 3. Schematic of the experimental design for colony reversibility. The starting algal
433 populations were taken from the Colony induction experiment (see Fig. 2) and transferred into fresh
434 medium.
435

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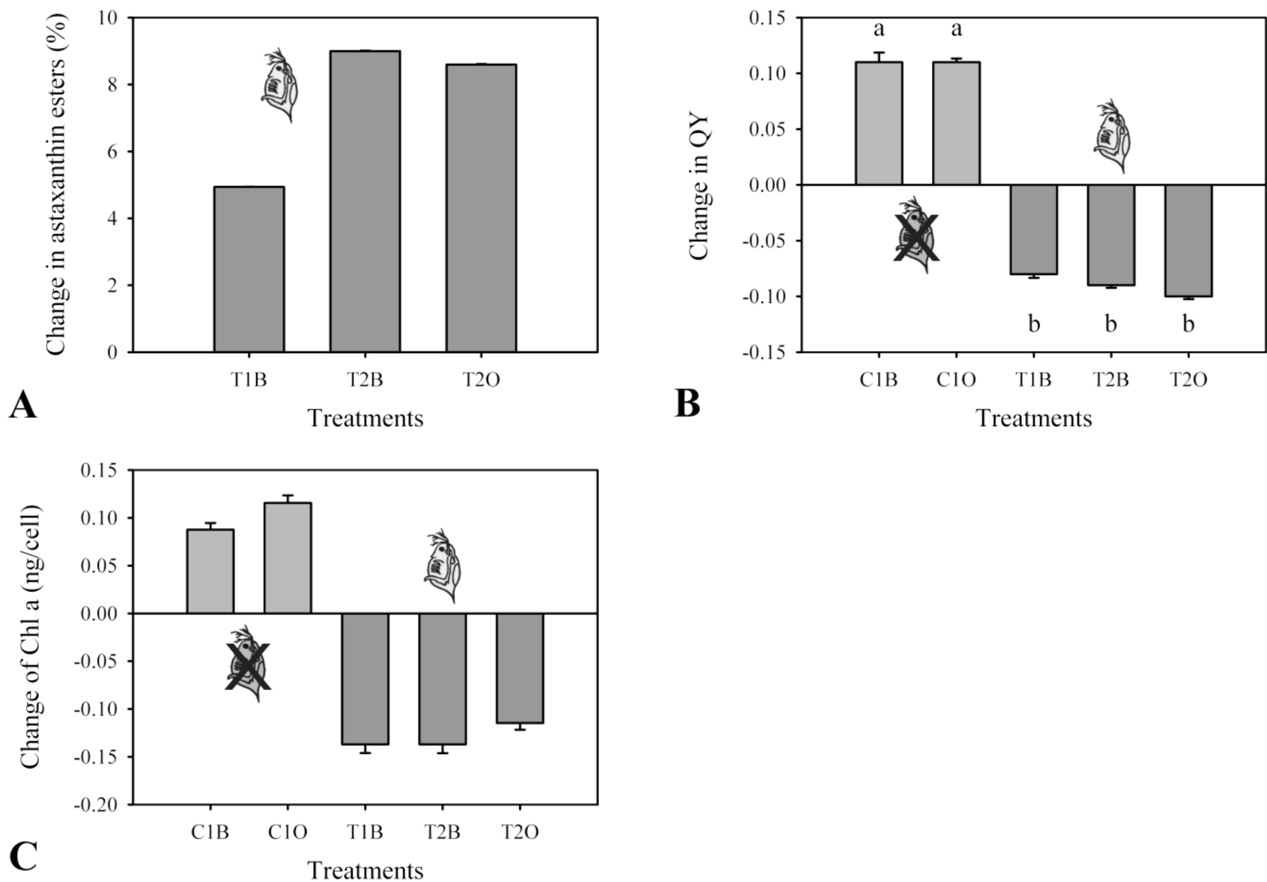


437

438 Figure 4. Colony induction experiment. A) Changes in % Astaxanthin esters in *Scenedesmus*
439 *obliquus* in the presence of *Daphnia magna*, between Day 0 and Day 3. Error bars represent the
440 standard errors. B) Changes in the quantum yield (QY, indicating PSII efficiency) of *S. obliquus*
441 between Day 0 and Day 3, in the presence or absence of *D. magna*. Error bars indicate standard
442 errors; replicates sharing the same letters are not statistically different. C) Changes in Chlorophyll *a*
443 (ng cell$^{-1}$) of *S. obliquus* between Day 0 and Day 3, in the presence or absence of *D. magna*. The
444 error bars represent standard errors. T = treatment, C = control, B=bag, O= outside, 1 and 2
445 represent the first and second treatments, respectively.

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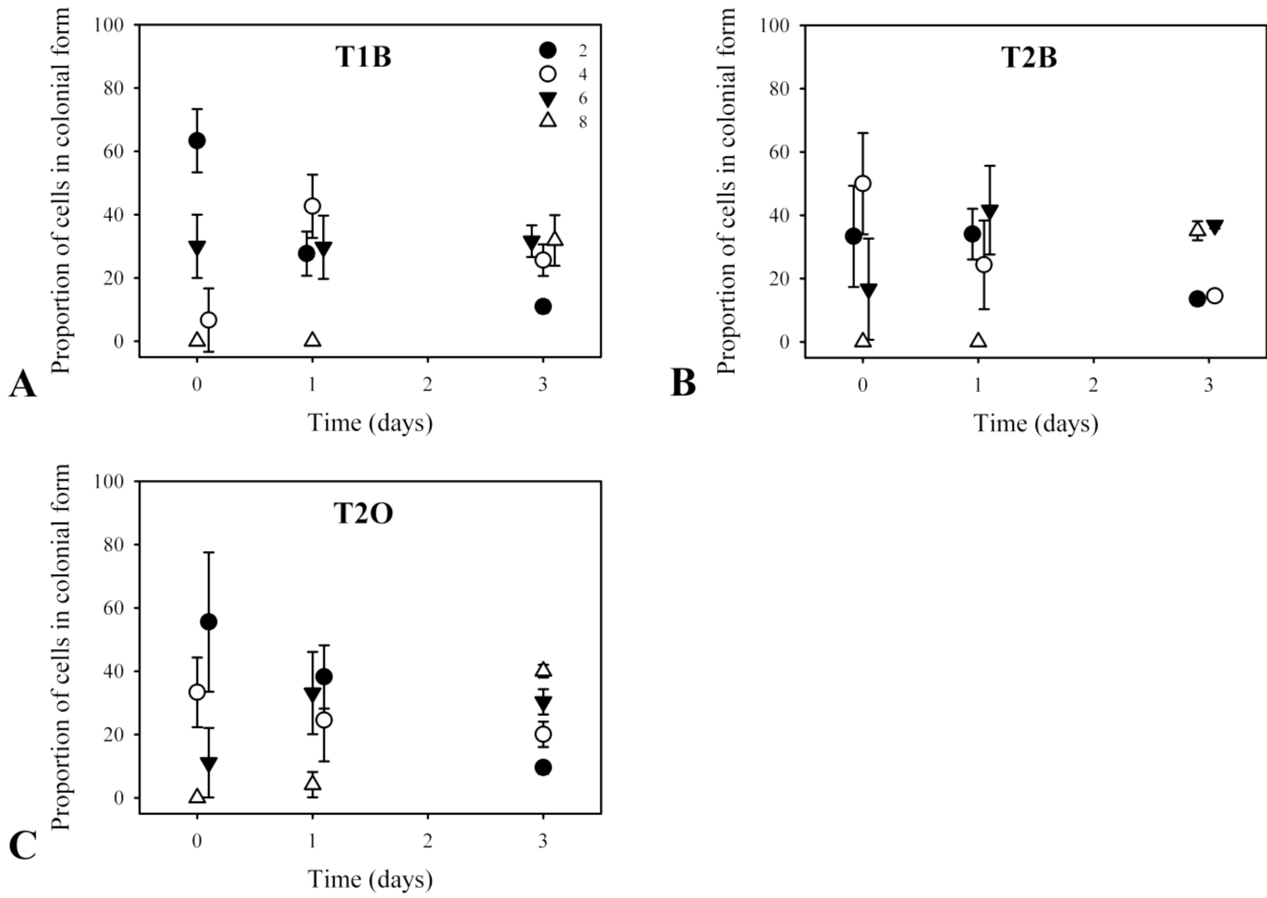


448

449 Figure 5. Colony induction experiment. Proportion of cells in colonial form of *Scenedesmus*
450 *obliquus* in the presence of *Daphnia magna* over time, for the different treatments. The error bars
451 represent the standard errors. See Fig. 2 for the treatment group notations.

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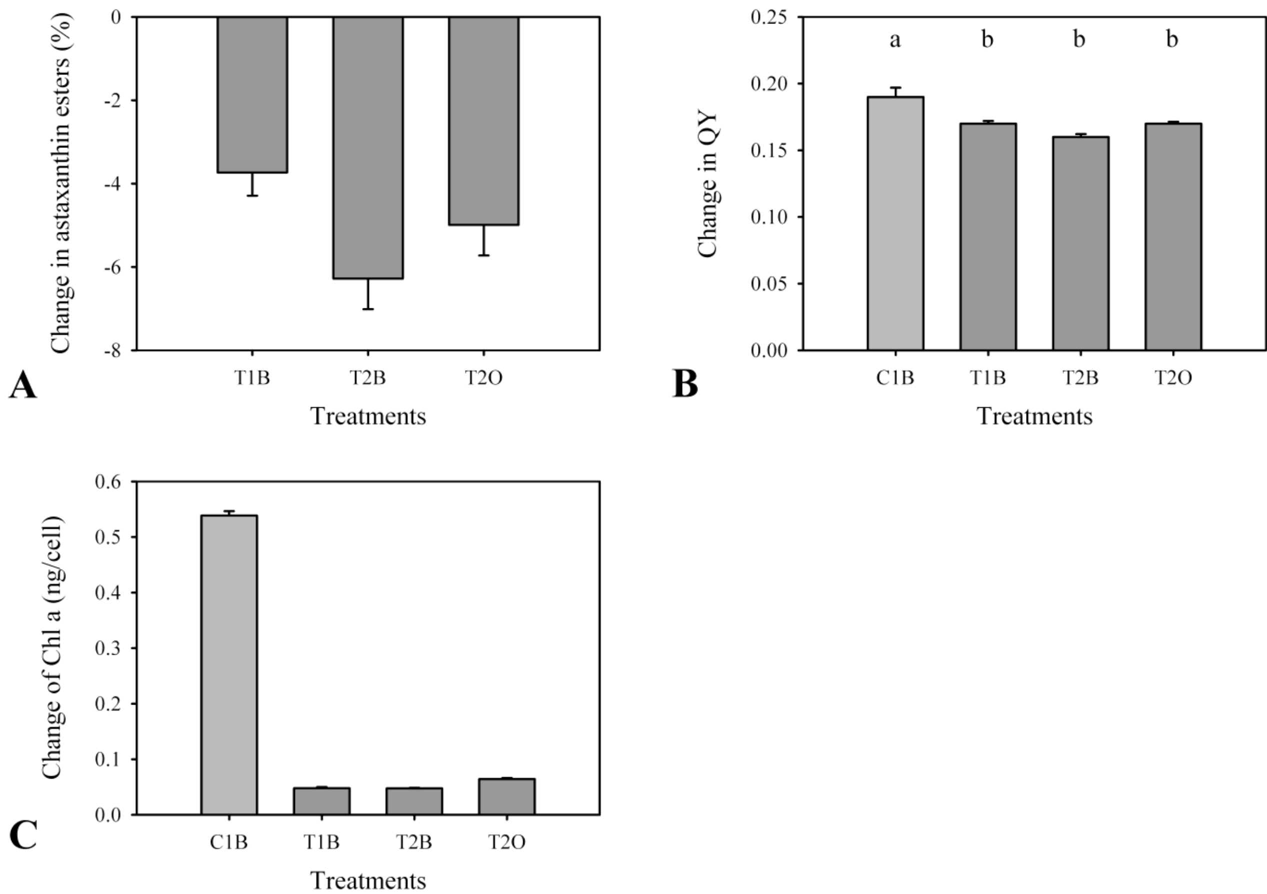


453

454 Figure 6. Colony reversibility experiment. A) Changes in % Astaxanthin esters in *Scenedesmus*
455 *obliquus* in the presence of *Daphnia magna*, between Day 0 and Day 3. B) Changes in the quantum
456 yield (QY, indicating PSII efficiency) of *S. obliquus* between Day 0 and Day 3, in the presence or
457 absence of *D. magna*. The error bars represent the standard errors; replicates sharing the same
458 letters are not statistically different. C) Changes in Chlorophyll *a* (ng $cell^{-1}$) of *S. obliquus* between
459 Day 0 and Day 3, in the presence or absence of *D. magna*. The error bars represent standard errors.
460 See Fig. 3 for the treatment group notations.

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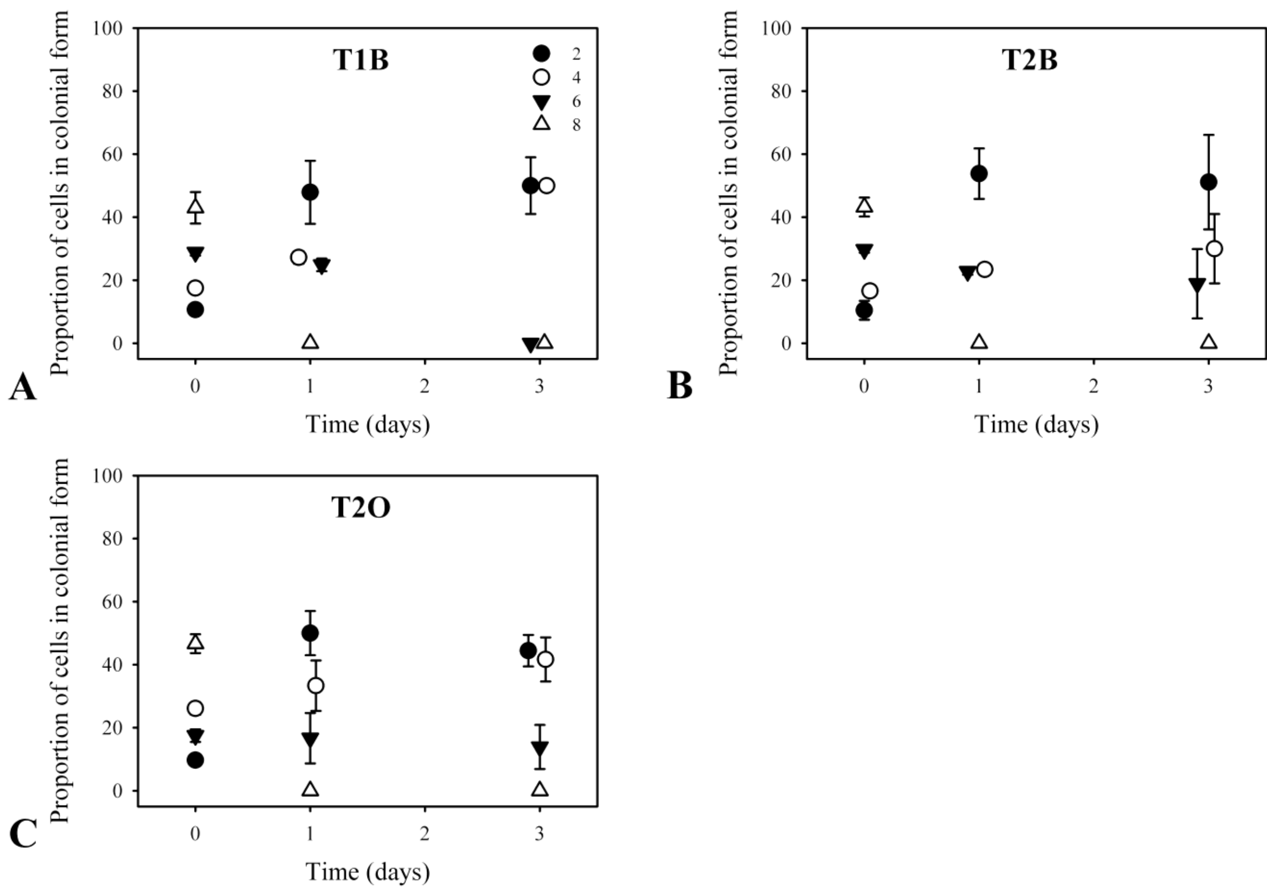


462

463 Figure 7. Proportion of cells in colonial form of *Scenedesmus obliquus* in the presence of *Daphnia*
464 *magna* over time, for the different treatments. The error bars represent the standard errors. See Fig.
465 2 for the treatment group notations.