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Multicellular group formation in response to predators in the alga *Chlorella vulgaris*

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Abstract

A key step in the evolution of multicellular organisms is the formation of cooperative multicellular groups. It has been suggested that predation pressure may promote multicellular group formation in some algae and bacteria, with cells forming groups to lower their chance of being eaten. We use the green alga *Chlorella vulgaris* and the protist *Tetrahymena thermophila* to test whether predation pressure can initiate the formation of colonies. We found that: (1) either predators or just predator exoproducts promote colony formation; (2) higher predator densities cause more colonies to form; and (3) colony formation in this system is facultative, with populations returning to being unicellular when the predation pressure is removed. These results provide empirical support for the hypothesis that predation pressure promotes multicellular group formation. The speed of the reversion of populations to unicellularity suggests that this response is due to phenotypic plasticity and not evolutionary change.

Introduction

Multicellularity has evolved over 25 times across the tree of life with species ranging from relatively simple organisms like photosynthetic cyanobacteria, to the most complex metazoans with hundreds of different cell types (Maynard Smith & Szathmáry, 1995; Grosberg & Strathmann, 2007). A problem here is that, given the possible fitness costs of forming groups, such as increased competition for resources, why would individual cells clump or cluster together (Lurling & Van Donk, 2000; Bourke, 2011; West *et al.*, 2015). One theory is that cells may clump together to defend themselves against predation (Stanley, 1973; Grosberg & Strathmann, 2007; Bourke, 2011; Claessen *et al.*, 2014). In support of this, several species of algae and bacteria have been shown to form clumps conditionally, in the presence of predators (Table 1).

There are, however, a number of potential complications with many of the previous studies examining group formation in algae. Some studies have focused on the number of colonies formed in the presence of predators, rather than the proportion of cells in colonies (Table 1). The number of colonies is confounded with population growth, and so does not necessarily reflect whether cells are more likely to form groups in the presence of predators. Another issues is that some studies lacked formal statistical analyses, or controls that tested for the consequences of adding nutrients with predators (Boraas *et al.*, 1998; Yang *et al.*, 2006; Yang & Li, 2007). Finally, there are a number of other issues that need to be addressed. For example, does the presence of more predators lead to greater colony formation, and do algae respond to direct (physical contact) or indirect (exoproducts) cues of predator presence (Stanley, 1973; Lampert *et al.*, 1994; Lurling & Van Donk, 1996; Ha *et al.*, 2004; Grosberg & Strathmann, 2007; Yang *et al.*, 2009; Bourke, 2011; Claessen *et al.*, 2014)? Another issue is whether colony formation is obligate, meaning cells consistently stick together regardless of environmental cues, or facultative, through phenotypic plasticity in response to local conditions (Boraas *et al.*, 1998; Lurling, 2009)? The distinction between obligate and facultative matters, because the formation of a heritable colony-forming phenotype

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Table 1 Group formation experiments on algae and bacteria.

Algae/bacteria species	Predator species	Measured proportion of cells in colonies?	Tested for indirect cues?	Reference
Cyanobium sp.	*Ochromonas* sp.	No	X	Jezberová & Komárková (2007)
Microcystis aeruginosa	*Ochromonas* sp.	No	✓	Yang *et al.* (2009)
	Daphnia magna	No	✓	Ha *et al.* (2004)
	Moina macrocopa	No	✓	Ha *et al.* (2004)
Phaeocystis	*Gyrodinium dominans*	No	X	Jakobsen & Tang (2002)
	Euplotes	Yes	X	Long *et al.* (2007)
Scenedesmus acutus	*Daphnia pulex*	No	✓	Lurling & Van Donk (1996)
	Daphnia galeata	Yes	✓	Lurling *et al.* (1997)
	Daphnia magna	Yes	✓	Lampert *et al.* (1994)
Scenedesmus subspicatus	*Daphnia magna*	No	✓	Lurling & Van Donk (1997)
Scenedesmus obliquus	*Daphnia magna*	No	✓	Lurling & Van Donk (1997)
Scenedesmus dimorphus	*Moina macrocopa*	No	✓	Ha *et al.* (2004)
Chlorella vulgaris	*Ochromonas vallescia*	No	X	Boraas *et al.* (1998)
Chlamydomonas reinhardtii	*Brachionus*	No	X	Becks *et al.* (2010)
Flectobacillus sp.	*Ochromonas* sp.	No	✓	Corno & Jurgens (2006)
Comamonas acidovorans	*Ochromonas* sp.	No	X	Hahn *et al.* (1999)

Shaded rows represent experiments on prokaryotes: *Microcystis aeruginosa* is a photosynthetic cyanobacteria (alga), *Flectobacillus* and *Comamonas* are nonphotosynthetic bacteria. The third column indicates whether the experiments measured the proportion of cells in colonies when predators were present, rather than potentially less informative measurements such as the number of colonies. ✓ indicates that the experiment tested for indirect cues of predation, such as waste products or signalling molecules, and X indicates that the experiment did not test for indirect cues of predation.

is a key step towards the evolution of an obligate multicellular organism.

We use the green alga *Chlorella vulgaris* and the protist predator *Tetrahymena thermophila,* to test whether the presence of a predator promotes colony formation. *Chlorella vulgaris* is an asexual freshwater green algae, found worldwide, that usually lives in unicellular populations, although clumping has been observed in laboratory strains on one occasion (Boraas *et al.*, 1998). *Tetrahymena thermophila*, also commonly found in freshwater habitats, is a free-living unicellular ciliate protist. If colony formation is a defensive response to predation, we would expect to see an increase in the proportion of cells in colonies when predators are present, a stronger response when the density of predators is high and possibly also a response to cues of predator presence, as well as to actual predators. We test these predictions by examining: (1) whether *T. thermophila* can induce colony formation in *C. vulgaris*; (2) whether *T. thermophila* exoproducts are sufficient to induce colony formation; (3) whether greater *T. thermophila* density leads to increased colony formation; and (4) whether colony formation is obligate or facultative, with cells returning to unicellular forms when predators are no longer around.

Materials and methods

In all experiments, we used the green algae species *C. vulgaris* and the ciliate protist *T. thermophila*. We purchased both as axenic cultures from the Culture Collection of Algae and Protozoa, strain numbers 211/11B and 1630/1M, respectively. We grew stock cultures of *C. vulgaris* at room temperature in 300 mL of Bolds Basal Media (Bolds) and kept them on a shaker to prevent sedimentation. We grew *T. thermophila* cultures at room temperature in 10 mL of Proteose Peptone Yeast Media (PPY) and subcultured stocks every week by transferring 100 μL to 10 mL of fresh PPY media. When PPY media is added to *C. vulgaris* cultures, the algae show an increased growth rate due to the high nutrient content of PPY. Consequently, we added sterile PPY media to algae-only control treatments, to control for the potential increase in growth rate.

To obtain independent measurements of colony formation each day for 5 days, we set up three replicates of each treatment (described in detail for each experiment below) and repeated these five times (three replicates of each treatment each day for 5 days). We then randomized these treatments in 24-well plates (1 mL volume per well). We therefore only measured each microcosm once, so that all measurements on consecutive days would be independent; that is, we did not have repeated measures. We kept the plates on a microplate shaker in a 20 °C incubator with a 16-h/8-h light/dark cycle.

We produced supernatants of: (1) predator-only culture; (2) algae-only culture; and (3) algae and predator culture. We produced the supernatant of predator culture by filtering *T. thermophila* in PPY media (400 000 cells mL^{-1}) through a 0.22-μm syringe filter, to remove all *T. thermophila* cells, leaving just a supernatant of exoproducts and PPY media. We produced

the algae supernatant using the same method described above, but with *C. vulgaris* in Bolds media. We produced the algae and predator supernatant by adding *T. thermophila* in PPY to *C. vulgaris* culture and then leaving this mixture for 1 h. We then filtered this through a 0.22-μm syringe filter to remove all cells, leaving only exoproducts and media. These different supernatants are referred to throughout as: 'predator supernatant' (supernatant of *T. thermophila* culture), 'algae supernatant' (supernatant of *C. vulgaris* culture), and 'algae & predator supernatant' (supernatant of *T. thermophila* and *C. vulgaris* culture).

In order to quantify the effect of predation on the algal populations, we captured images of the experimental cultures using a VisiCam on an inverted light microscope and processed the images using IMAGEJ software (National Institute of Mental Health, Bethesda, MD, USA). In all experiments, we took one photograph of each relevant microcosm (corresponding to the day of the experiment – one, two, etc.) at 20× magnification, focusing on a random area of the well. We then used IMAGEJ software to manually count the number of unicells and the number of colonies in each photograph, using the CellCounter plugin. To ensure the quantification was blind, we firstly assigned each photograph a random code rather than a filename including treatment names and days, and secondly deferred all counting until after the experiment was complete. We defined three or more cells with cell walls touching to be colonies, on the basis that this is simplest definition of a multicellular phenotype. Where possible, we estimated the size of each colony and calculated mean colony size (for some colonies, this was not possible due to poor image quality). If cells appeared to be paired, we counted them as two unicells.

Experiment 1: do predators induce colony formation?

We tested whether the timing and number of times that *T. thermophila* was added to algal populations had any influence on colony formation. The experiment had three treatments: (1) the addition of *T. thermophila* to *C. vulgaris* on day one only (p1); (2) the addition of *T. thermophila* to *C. vulgaris* every day for days one to five (p1–5); and (3) the addition of *T. thermophila* to *C. vulgaris* on day 3 (p3). As a control, we used *C. vulgaris* in PPY media. We replicated each treatment 15 times, giving a total of $4 \times 15 = 60$ microcosms (replicates), which we randomized in 24-well plates.

Experiment 2: do predator exoproducts induce colony formation?

We tested whether colony formation occurred in the presence of *T. thermophila* exoproducts.

The experiment had four treatments: (1) the addition of 50 μL *T. thermophila* (roughly 10 000 individuals) in PPY media to 950 μL *C. vulgaris*, (2) addition of 50 μL *T. thermophila* supernatant to 950 μL *C. vulgaris*, (3) addition of *T. thermophila* and *C. vulgaris* supernatant to 950 μL *C. vulgaris* and (4) addition of 50 μL *C. vulgaris* supernatant to 950 μL *C. vulgaris*. We added the live predator treatment, as we wanted to control for potential differences with Experiment 1. We had two controls; (1) 1 mL *C. vulgaris* in Bolds media and (2) 950 μL *C. vulgaris* with 50 μL PPY media (to control for the increased growth in high nutrient PPY media). We replicated each treatment 15 times, giving a total of $6 \times 15 = 90$ microcosms, which we randomized in 24-well plates.

Experiment 3: does predator density influence colony formation?

We tested whether *T. thermophila* density affected the strength of colony formation in *C. vulgaris*. The experiment had three treatments: (1) low density of *T. thermophila* (11 000 cells mL^{-1}), (2) medium density of *T. thermophila* (31 000 cells mL^{-1}) and (3) high density of *T. thermophila* (1 000 000 cells mL^{-1}), all of which were added to *C. vulgaris* culture. As a control, we used *C. vulgaris* in PPY media. We replicated each treatment 15 times, giving a total of $4 \times 15 = 60$ microcosms, which we randomized in 24-well plates.

Experiment 4: is colony formation facultative?

This experiment was an extension of Experiment 1. We tested whether colony formation in *C. vulgaris* is obligate or facultative. We kept Experiment 1 plates in the incubator for an extra 15 days without any additional *T. thermophila*, and we transferred to fresh media on day 10. On day 20, we took photographs of the microcosms and quantified colony formation using the same protocol described above.

Statistical methods

We used R version 2.14.1 for statistical analysis (R Foundation for Statistical Computing, Vienna, Austria). We used generalized linear models (glm() command) and specified error structures (family =) as quasi-Poisson for number of colonies and total number of cells, and quasi-binomial for proportion of cells in colonies to account for overdispersion in the data, and Gaussian for colony size. In all analyses, the treatment (e.g. density of predators) and day were used as fixed effects, and the response variable was either the number of colonies, the proportion of cells in colonies, mean colony size or total number of algal cells. To test for differences between each treatment, we used multiple comparison of means ('multcomp' package, glht() command specifying Tukey contrasts). We only tested for differences between means on relevant days; for example, the day after predators had been added or on the

final day of the experiment, as we were interested in the immediate effect of predators or predator exoproducts on colony formation and not the overall population dynamics of algal cultures.

Results

Experiment 1: do predators induce colony formation?

We found that the effect of predation treatment on the proportion of cells in colonies was significant (GLM, $F_{3,56} = 25.60$, $P < 0.001$), whereas the effect of day and the interaction between predation treatment and day had no significant effect (day: $F_{1,55} = 0.99$, $P = 0.33$; treatment × day: $F_{3,52} = 1.74$, $P = 0.17$). By day 3, the treatments where predators had been added on day 1 (p1 and p1–5) had a significantly higher proportion of cells in colonies than treatments where predators had not been added (control and p3), (control vs. p1: $t = 6.91$, $P = 0.0001$; control vs. p1–5: $t = 8.35$, $P < 0.001$; control vs. p3: $t = 1.36$, $P = 0.21$; overall difference between all treatments: $F_{3,8} = 57.33$, $P = 0.001$) (Figs 1 and 2a). This corresponded to an increase from < 10% to > 60% of cells being part of a colony when predators were continually added (p1–5). On day 5, only the treatment when predators had been continually added (p1–5) had a significantly higher proportion of cells in colonies compared with the control (p1: $t = 1.90$, $P = 0.09$; p1–5: $t = 3.20$, $P = 0.01$; p3: $t = 2.02$, $P = 0.08$; overall difference between all treatments: $F_{3,8} = 10.78$, $P = 0.003$).

We found a similar pattern for the number of colonies (Fig. 2b). We found that the effect of both predation treatment and the day of the experiment on the number of colonies was significant (predation treatment: GLM, $F_{3,56} = 35.20$, $P < 0.001$; day: $F_{1,55} = 72.79$, $P < 0.001$; treatment × day: $F_{3,52} = 3.79$, $P = 0.02$). By day 3, treatments where predators had been added on day 1 (p1 and p1–5) had significantly more colonies than treatments where predators had not been added (control and p3) (control vs. p1: $t = 3.62$, $P = 0.007$; control vs. p1–5: $t = 4.20$, $P = 0.003$; control vs. p3: $t = 0.81$, $P = 0.44$; overall difference between all treatments: $F_{3,8} = 16.81$, $P = 0.001$). On day 5, all treatments where predators had at some point been added had significantly more colonies compared to the control where predators had not been added (control vs. p1: $t = 3.45$, $P = 0.009$; control vs. p1–5: $t = 5.51$, $P = 0.001$; control vs. p3: $t = 3.28$, $P = 0.01$; overall difference between all treatments: $F_{3,8} = 31.05$, $P = 0.001$). When we added predators continually (p1–5), this led to significantly more colonies than when we just added predators on day 1 or three (p1 and p3) and all other treatments (multiple comparisons of means, p1–5 vs. p1: $z = 4.88$, $P < 0.001$; p1–5 vs. p3: $z = -5.11$, $P < 0.001$). Overall, we found that mean colony size was not affected by the addition of predators or by the day of the experiment (predation treatment: GLM, $F_{3,56} = 0.17$, $P = 0.92$; day: $F_{1,55} = 1.36$, $P = 0.25$; predation treatment × day: $F_{3,52} = 1.67$, $P = 0.19$). The total number of algal cells was affected by the addition of predators and the day of the experiment (predation treatment: GLM, $F_{3,56} = 6.17$, $P = 0.001$; day: $F_{1,55} = 145.01$, $P < 0.001$), but the interaction was nonsignificant ($F_{3,52} = 1.86$, $P = 0.15$) (Fig. 2c).

Experiment 2: do predator exoproducts induce colony formation?

We found a significant effect of the addition of supernatants and the day of the experiment (supernatant treatment: GLM, $F_{5,84} = 7.82$, $P < 0.001$; day: $F_{1,83} = 21.44$, $P < 0.001$), with the interaction being nonsignificant ($F_{5,78} = 2.05$, $P = 0.08$). Looking at specific days, we found no difference in the proportion of cells in colonies between 'predator-related' and control treatments on day 1 (GLM, $F_{1,16} = 0.60$, $P = 0.45$). On day 3, we found that 'predator-related' treatments (predator + algae supernatant, predator supernatant and live predators) did not have a significantly higher

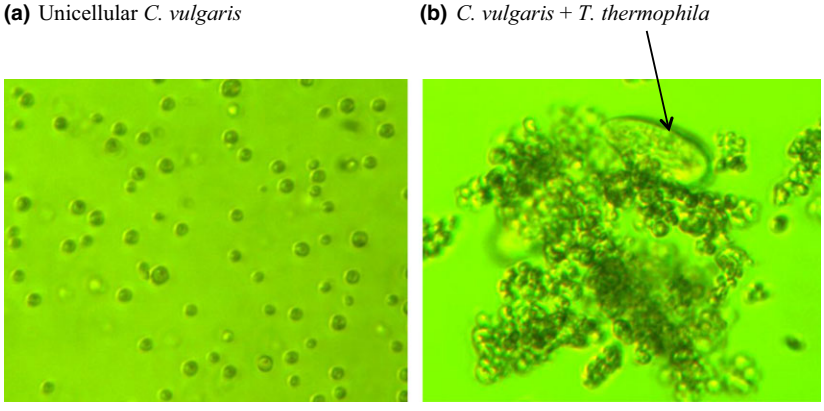

Fig. 1 Examples of *Chlorella vulgaris* and *Tetrahymena thermophila* cultures. (a) *C. vulgaris* unicells when predators are absent. (b) The formation of colonies when predators are added (a particularly large colony is shown for illustrative purposes). An individual *T. thermophila* protist can be seen in image (b), indicated with an arrow. Both images were taken using VISICAM software at 40× magnification.

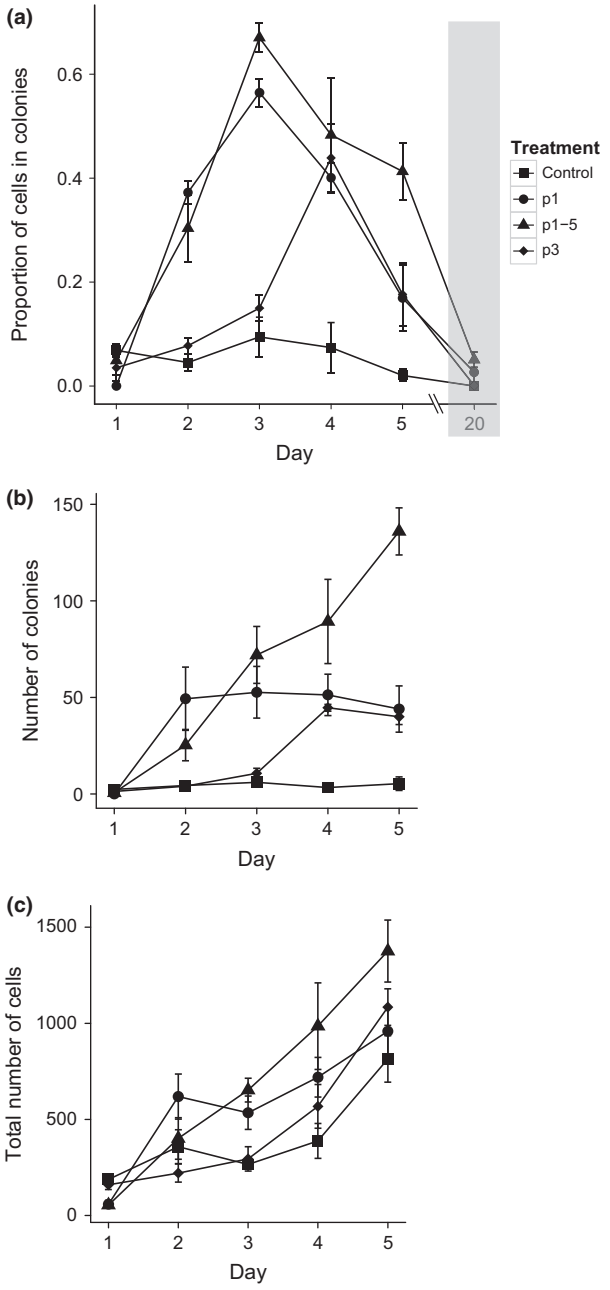

Fig. 2 Predators induce facultative colony formation. Plots showing (a) the proportion of cells in colonies showing days one to 20, (b) the number of colonies, (c) total number of algal cells for all treatments. Treatments were p1: predators added on day 1, p1–5: predators added every day, p3: predators added on day 3, control: no predators added. $N = 3$ for each treatment on each day. The top legend applies to all figures. Means and standard errors are shown.

proportion of cells in colonies compared with control treatments (algae, PPY and algae supernatant) (GLM, $F_{1,16} = 4.42$, $P = 0.05$) (Fig. 3a). However, on day 5, we found that 'predator-related' treatments had significantly higher proportion of cells in colonies than control treatments (GLM, $F_{1,16} = 10.16$, $P = 0.006$). This result remained the same even when we removed the live predator treatment: 'predator-related' treatments had a significantly higher proportion of cells in colonies compared with control treatments (GLM, $F_{1,13} = 7.08$, $P = 0.02$). We found the same results when we compared the 'predator-related' treatments to just the PPY control on day 5 ($F_{1,16} = 5.61$, $P = 0.04$), but not day 3 ($F_{1,16} = 1.42$, $P = 0.26$). Considering the number of colonies, we found a significant effect of supernatant treatment and the day of the experiment (supernatant treatment: GLM, $F_{5,84} = 15.74$, $P < 0.001$; day: $F_{1,83} = 49.65$, $P < 0.001$). We found that 'predator-related' treatments had significantly more colonies compared with the control treatments on both day 3 (GLM, $F_{1,16} = 8.89$, $P = 0.009$) and day 5 (GLM, $F_{1,16} = 15.23$, $P = 0.001$) (Fig. 3b). We found no significant difference in mean colony size between supernatant treatments and the control (GLM, $F_{5,84} = 0.47$, $P = 0.80$).

Experiment 3: does predator density influence colony formation?

We found that both predator density and the day of the experiment had a significant effect on the proportion of cells in colonies (predator treatment: GLM, $F_{3,56} = 11.68$, $P < 0.001$; day: $F_{1,55} = 25.49$, $P < 0.001$; predator treatment × day: $F_{3,52} = 3.34$, $P = 0.03$). There was a significantly higher proportion of cells in colonies when predator density was higher, examining days three (GLM, $F_{1,11} = 9.88$, $P = 0.01$), four (GLM, $F_{1,11} = 10.76$, $P = 0.008$) and five (GLM, $F_{1,11} = 10.19$, $P = 0.01$) (Fig. 4). When predator density was high, this resulted in >50% cells in colonies on day 5, compared with <30% when no predators were present. We also found a significant interaction between predator density and the day of the experiment (GLM, $F_{3,52} = 3.34$, $P = 0.03$), meaning that the effect of predator density on the proportion of cells in colonies depended on the day. In contrast, there was no significant difference in mean colony size between different predator densities (GLM, $F_{3,52} = 0.25$, $P = 0.86$).

Experiment 4: is colony formation facultative?

By day 20, we found no difference in the proportion of cells in colonies between the treatments where predators had been added compared with the treatment where no predators had been added (control vs. p1: $t = 0.004$, $P = 1.00$; control vs. p1–5: $t = 0.004$, $P = 1.00$; control vs. p3: $t = 0.003$, $P = 1.00$; overall difference in proportion of cells in colonies between all treatments: $F_{3,8} = 6.19$, $P = 0.02$) (Fig. 5a).

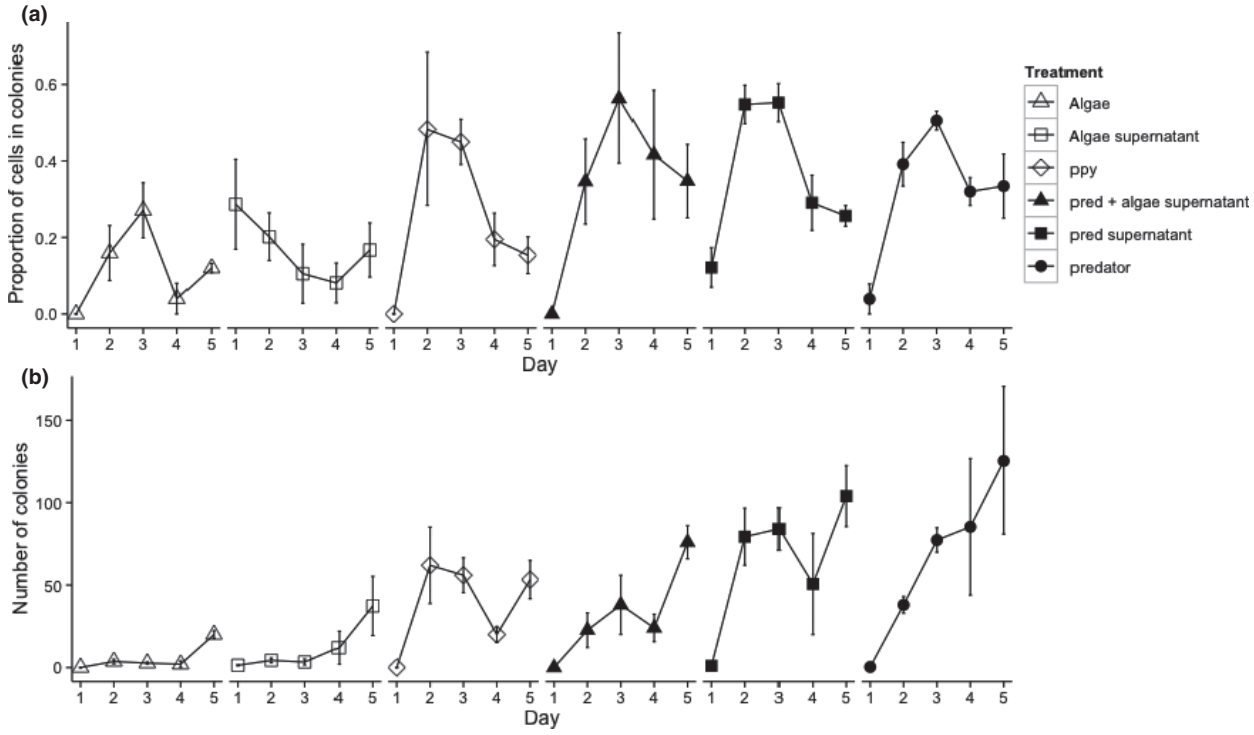

Fig. 3 Predator exoproducts induce colony formation. (a) The proportion of cells in colonies and (b) the number of colonies. 'Predator-'related treatments (live predators, predator supernatant and predator + algae supernatant) are shown with filled shapes, and 'control' treatments (algae, algae supernatant and algae + PPY media) are shown with open shapes. $N = 3$ for each treatment on each day. The right hand legend applies to both figures. Means and standard error bars are shown.

Discussion

We found that: (1) the presence of the protist predator *T. thermophila* promotes colony formation in the algae *C. vulgaris* (Figs 1 and 2); (2) supernatants taken from predator cultures and predator/algae cultures are able to promote colony formation (Fig. 3); (3) higher predator densities cause more cells to form colonies (Fig. 4); and (4) colony formation in this system is facultative, with populations reverting to being predominantly unicellular after 20 days, when there were no live, mobile predators left in the culture (Fig. 5).

We have shown that *T. thermophila* can promote colony formation in *C. vulgaris* (Fig. 1) and that predator and predator/algae exoproducts are sufficient to induce this response (Fig. 3). It has previously been observed that the presence of *Ochromonas vallescia* causes colony formation in *C. vulgaris* (Boraas *et al.*, 1998). We have shown experimentally that *C. vulgaris* forms colonies in response to not only live predators, but also predator and predator/algae exoproducts and that a higher density of predators causes more colonies to form (Figs 1 and 4). Previous studies have observed that predator exoproducts promote colony formation in other green algae, including *Microcystis, Scenedesmus, Phaeocystis* and *Chlamydomonas* (Hessen & Van Donk, 1993; Lurling & Van Donk, 1997; Tang, 2003; Ha *et al.*, 2004; Yang *et al.*, 2009; Becks *et al.*, 2010) (Table 1). Detecting predator exoproducts (e.g. waste products, pheromones) could be a quick and reliable way for algae to respond to the presence of specific predators, before the predators are actually a direct threat (Van Donk *et al.*, 2011). Our results are consistent with the hypothesis that colony formation is a defensive response to the presence of predators (Mayeli *et al.*, 2005; Van Donk *et al.*, 2011; Claessen *et al.*, 2014) and hence that predation pressure could be important for the evolution of cooperative multicellular groups. This is analogous to the formation of social groups in animals as a predator defence (Foster, 1990; Grosberg & Strathmann, 2007; Pike *et al.*, 2007; Bourke, 2011; Shultz *et al.*, 2011; Korb *et al.*, 2012).

Predation pressure is not the only reason cells may form multicellular groups. Being part of a multicellular group could increase local cell density in a way that allows more efficient use of extracellular products, as has been argued in bacteria and yeast (Rosenberg *et al.*, 1977; Koschwanez *et al.*, 2011; Darch *et al.*, 2012; Biernaskie & West, 2015). Another possibility is that multicellular fruiting bodies may allow for more efficient

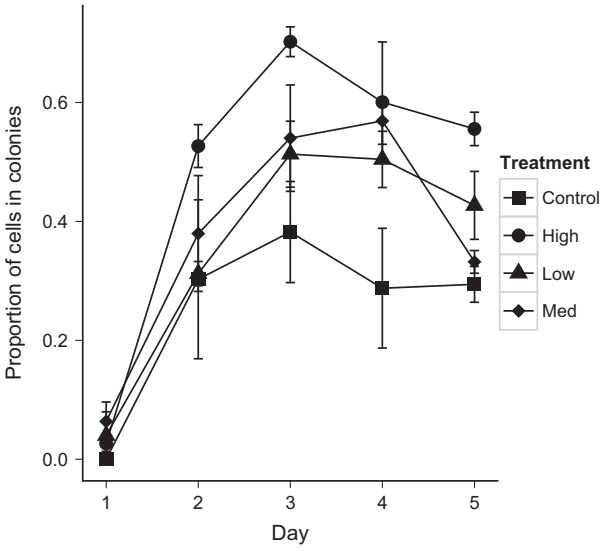

Fig. 4 Predator density influences the proportion of cells in colonies. The three treatments were: low density of *Tetrahymena thermophila* (11 000 cells mL^{-1}), medium density of *T. thermophila* (31 000 cells mL^{-1}) and high density of *T. thermophila* (1 000 000 cells mL^{-1}) and the control was *Chlorella vulgaris* in PPY media. $N = 3$ for each treatment on each day. Means and standard errors are shown.

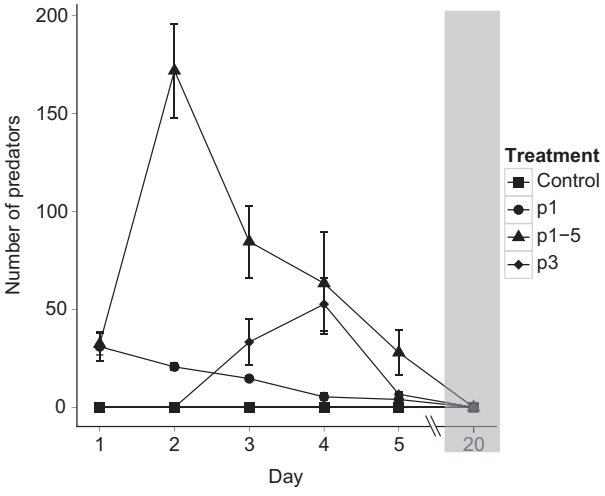

Fig. 5 Predator populations decrease over 20 days. The number of live, mobile predators (per field of view at 10× magnification) for all treatments (p1: predators added on day 1, p1–5: predators added every day, p3: predators added on day 3, control: no predators added) over the course of 20 days. $N = 3$ for each treatment on each day. Means and standard errors are shown.

dispersal, as has been argued in *Dictyostelium* slime moulds and *Myxococcus* bacteria (Bonner, 1967; Kaiser, 1986; Velicer & Yu, 2003; Gilbert *et al.*, 2007). Recent experimental studies have also shown that colony formation can be favoured by artificial selection, with both the alga *Chlamydomonas reinhardtii* and the yeast *Saccharomyces cerevisiae* forming obligate multicellular clusters under intense selection for large clusters through centrifugation and settling (Ratcliff *et al.*, 2012, 2013). These experiments show that, in artificial selection regimes, the formation of multicellular clusters is possible in a relatively short time frame.

We found that the presence of predators had no significant influence on mean colony size. One possible explanation is that there is an optimum colony size, for a given environment. For example, if competition for resources led to an increasing cost of forming larger colonies (Yang *et al.*, 2009), then this would have to be traded off against any increased ability to defend against predators (Lurling & Van Donk, 2000). Some predators of algae are size-selective (Long *et al.*, 2007), and so the benefits of increased colony size could also asymptote with colony size.

We observed that the total number of *C. vulgaris* cells did not significantly vary depending upon whether *T. thermophila* were present or not (Fig. 1c). There are at least three possible explanations for this. First, the predator densities that we used, while sufficient to trigger colony formation, were not sufficient to impact on population growth. Second, the increased colony formation was able to prevent appreciable predation. Third, that *T. thermophila* is a poor predator of *C. vulgaris*. The first and third of these possibilities would indicate a generic response by *C. vulgaris* to potential predators in its local environment (potential predation), rather than the specific local danger of predation.

We have shown that in our experimental set-up, colony formation in *C. vulgaris* is facultative, with populations reverting back to being predominantly unicellular when live predators are absent (Fig. 5). In contrast to our result, Boraas *et al.* (1998) found that *C. vulgaris* colonies persisted for several months even after predators were removed. There are multiple differences between our experiments that could explain this difference. For example, Boraas *et al.* (1998) used a different predator, the flagellate protist *O. vallescia*, which could have led to a different selection pressure. Another possibility is that their experimental system used 500 mL chemostat cultures, compared with ours, which used volumes of 1 mL, which could have led to different costs or benefits of group formation.

Our results cannot exclude the possibility that the increased proportion of cells in colonies is a response to selection for a fixed colony-forming phenotype and that the reversion to a predominantly unicellular population is a response to selection for unicells. In our experimental set-up, *C. vulgaris* has a doubling time of roughly 4 h, so it is possible that over five days (roughly 30 generations) we could potentially observe experimental evolution of multicellular groups. However, this is a small number of generations to see such

a large response due to genetic change. Furthermore, experimental evolution in *Chlamydomonas*, a related species of green algae (Ratcliff *et al*., 2013), has shown that multicellular clusters take over 200 days to evolve (>300 generations), even under intense selection regimes, so it is unlikely that we are seeing such rapid evolution over 5 days. Facultative colony formation may be favoured over the formation of obligate colonies, because it allows algae to have a flexible response to different types of predators (Van Donk *et al*., 2011), particularly if the benefit of colony formation only outweighs the cost in specific environments. The next steps will be to investigate the costs and benefits of group formation in this system and clarify how multicellular groups form.

To conclude, we have focused on the formation of multicellular groups, which is just the first stage in the evolution of a multicellular organism (Maynard Smith & Szathmáry, 1995; Bourke, 2011; West *et al*., 2015). The next step is cells differentiating into a variety of types, producing a division of labour (Gavrilets, 2010). Examples of this in simple multicellular organisms include the division between sterile stalk cells and reproductive spore cells in the fruiting bodies of *Dictyostelium* slime moulds and *Myxococcus* bacteria, and the division between motile somatic cells and non-motile germ cells in the Volvocine algae (Michod, 2007; Herron & Michod, 2008; Hanschen *et al*., 2014).

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