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Predation and the formation of multicellular groups in algae

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ABSTRACT

Background: The evolution of multicellular organisms must, at some point, have involved the congregating of single-celled organisms. Algal species exist that sometimes live in groups and sometimes live as single cells. Understanding the conditions that lead to algal assemblage in such cases may cast light on the selective forces that favour multicellularity.

Hypothesis: Forming groups could defend algae against predation if predators are unable to engulf large-sized entities.

Organisms: Three algal prey (*Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus*) and three predators (*Ochromonas* spp., *Tetrahymena thermophila*, and *Daphnia magna*).

Methods: We tested the tendency to aggregate in all nine different prey–predator combinations.

Results: At least two of the predators, *Ochromonas* and *Daphnia*, were significant predators because their presence decreased algal density. In all nine combinations, adding the predator species led to the formation of algal groups. In three combinations, adding merely products of the predators in the absence of the predators themselves stimulated group formation.

Keywords: Chlorophyceae, group formation, group size, induced defence, multicellularity.

INTRODUCTION

The tree of life can be viewed as a hierarchy of major evolutionary transitions in individuality (Maynard Smith and Szathmary, 1995; Bourke, 2011; West *et al.*, 2015). In each of these transitions, a group of individuals that could previously replicate independently formed a mutually dependent cooperative group. For example, genes formed genomes and cells formed multicellular organisms. Major evolutionary transitions can be divided into two steps: the formation of a cooperative group, and then the transformation of that group into a new higher level of individual (Bourke, 2011; West *et al.*, 2015). The major transitions approach emphasizes that classic problems in the study of evolutionary ecology, such as group formation and cooperation, are fundamental to understanding the development of complex life on Earth (Davies *et al.*, 2012).

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We focus on group formation in the transition from single-celled to multicellular life. A number of ecological factors have been suggested to drive the formation of multicellular groups (Grosberg and Strathmann, 2007; Claessen *et al.*, 2014). Groups may be able to make more efficient use of extracellular factors, such as the invertase produced by the yeast *Saccharomyces cerevisiae* to break down sugars (Koschwanez *et al.*, 2011, 2013; Biernaskie and West, 2015). Cooperative groups may be better able to disperse, as illustrated by the fruiting bodies of *Dictyostelium* slime moulds (Smith *et al.*, 2014) and *Myxococcus* bacteria (Velicer and Yuen-tsu, 2003). Groups may be better able to store resources, allowing individuals to cannibalize group-mates under conditions of starvation (Kerszberg and Wolpert, 1998; Raven, 1998; Szathmáry and Wolpert, 2003). Groups may also be better at predating (Dworkin and Bonner, 1972; Nichols *et al.*, 2009; Roper *et al.*, 2013), such as 'wolf-pack feeding' in myxobacteria (Dworkin and Bonner, 1972; Berleman and Kirby, 2009). Finally, defence against predation has been argued to favour the formation of groups, in algae and bacteria, because predators could have problems engulfing larger-sized entities (Stanley, 1973; Boraas *et al.*, 1998; Grosberg and Strathmann, 2007; Claessen *et al.*, 2014).

We examined the response of three freshwater unicellular Chlorophyte algal species (*Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus*) to three predatory species (the flagellate *Ochromonas* spp., the ciliate *Tetrahymena thermophila*, and the crustacean *Daphnia magna*) (Fig. 1). Our aims were to examine the generality and nature of the response to predators, and to use our results to test the utility of different species combinations for studying the evolutionary ecology of group formation in algae. In these nine 'algal–putative predator' combinations, we measured the influence of adding live predators on the proportion of algal cells in groups, the mean group size, and algal density. We complemented these experiments with behavioural observations to determine the extent to which the putative predators were actually predating the algal species.

The addition of predators could lead to the formation of groups for three broad reasons. First, individuals could facultatively form groups in response to the presence of predators. Second, the extent of group formation could be a fixed strategy, but by preferentially feeding on smaller groups, predators adjust the group size distribution. Third, the presence and movement of predators could move the algae into each other, and hence produce clumps. We distinguished the first possibility from the other two by examining whether the products of predators stimulate group formation in the absence of actual predators. This also requires that the algae use predator products as cues of the presence of predators (Lampert *et al.*, 1994; Yasumoto *et al.*, 2005; Uchida *et al.*, 2008; Fisher *et al.*, 2016).

MATERIALS AND METHODS

Species and growth conditions

Algae. We grew *Chlorella vulgaris* (axenic from CCAP; strain number 211/11B), *Chlorella sorokiniana* (non-axenic from CCAP; strain number 211/8K), and *Scenedesmus obliquus* (non-axenic from CCAP; strain number 276/3A) cultures in Bolds Basal media at a light/dark cycle of 16:8 hours. We treated 1-mL samples from the non-axenic cultures with 500 $\mu g \cdot mL^{-1}$ of the antibiotic rifampicin (a concentration that inhibited bacterial growth on KB agar plates). After 24 hours, we diluted these algal cultures 1:300 in Bolds Basal media and left them to grow in a 1-litre Erlenmeyer flask with shaking at 220 rpm and 22°C for at least a week prior to each experiment. We maintained the algae in all three cultures in a unicellular state at a density of $\sim 10^6$ cells $\cdot mL^{-1}$.

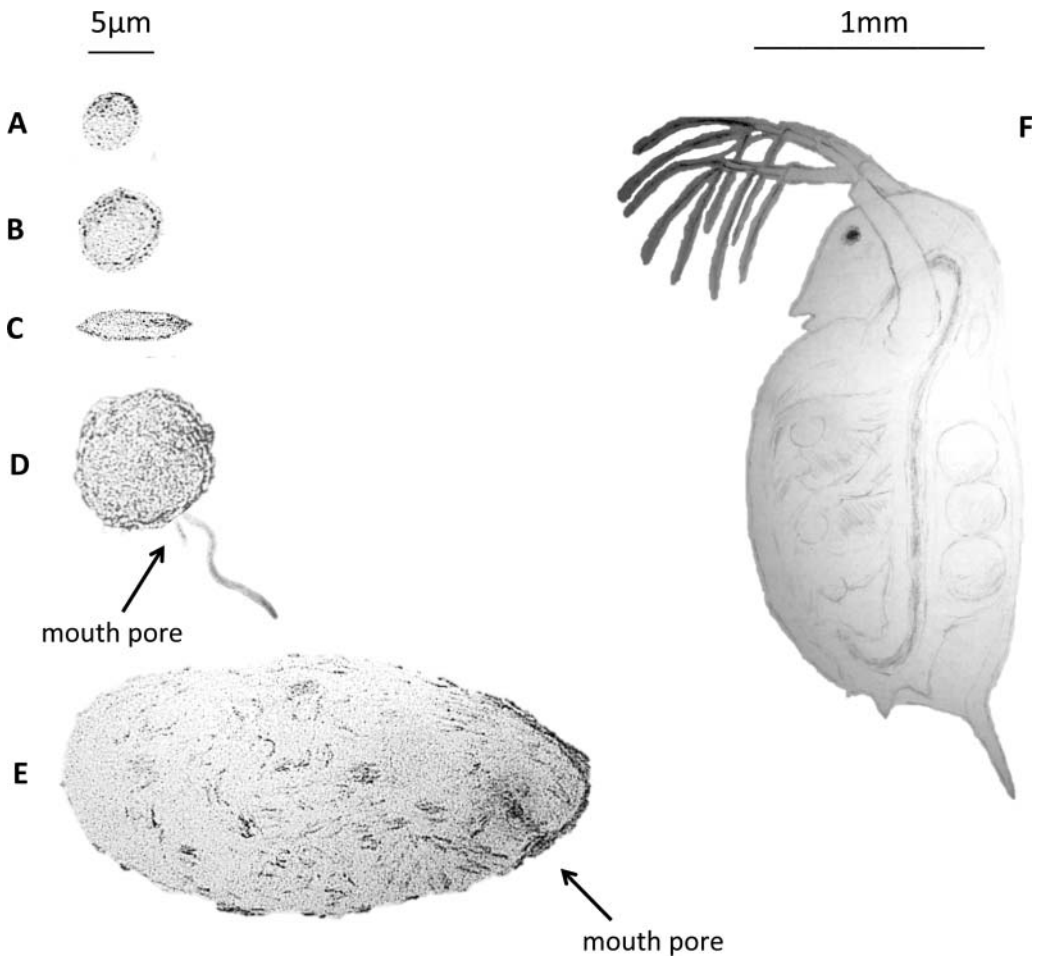

Fig. 1. The species used in the present study: (A) *Chlorella sorokiniana*, (B) *Chlorella vulgaris*, (C) *Scenedesmus obliquus*, (D) the flagellate *Ochromonas*, (E) the ciliate *Tetrahymena thermophila*, and (F) the crustacean *Daphnia magna*.

Protists. We grew *Tetrahymena thermophila* (axenic from CCAP; strain number 1630/1M) in Proteose Peptone Yeast extract (PPY) media in 20-mL flat-bottomed flasks at 25°C and a light/dark cycle of 16:8 hours. We grew *Ochromonas* spp. (from Corno and Jürgens, 2006) in PPY media in the dark.

Daphnia. We cultured *Daphnia magna*, obtained from a local fish store (The Goldfish Bowl, Oxford), in 1-litre jars with Tetra flake food at room temperature and constant air flow to allow for oxygenation.

Experiment 1: Testing for group formation and size change upon exposure to predators

We tested whether the addition of a predator led to the algae being more likely to form groups and/or increase their group size. For the *Chlorella sorokiniana–Ochromonas* spp. combination, we used thirty 50-mL falcon tubes (www.evolutionary-ecology.com/data/3034Appendix.pdf, Fig. S1). In each tube, we added 19.6 mL of *C. sorokiniana* to either 0.4 mL of PPY in the control or 0.4 mL of *Ochromonas* spp. in the treatments. We incubated the tubes at 20°C and a light/dark cycle of 16:8 hours using fluorescent illumination and kept the tube caps loose to allow for oxygenation. We randomized the tubes on tube racks to take into account any possible variation in treatments, such as position-derived differences in exposure to light.

We collected samples at four time points after adding the putative predator: at 1, 24, 48, and 72 hours. On each occasion, we tilted the falcon tubes five times, to adequately mix the cultures, and transferred 200 μL of each culture into a 96-well plate. We minimized any possibility of sampling error by obtaining an image from the centre of each well with a VisiCam digital camera under an inverted microscope (VWR, Model XDS-3) at 20× magnification. We performed image analysis by 'blind counting', where we did not know whether we were counting a treatment or a control well, to minimize bias. We quantified the proportion of cells in groups (number of algal cells in groups/total number of algal cells) and the mean group size. We define a group as ≥ 3 cells in contact with each other. The experimental procedure was the same for the remaining combinations, except for variation in the concentrations of algae and putative predators, the total volume used per tube, and the number of independent replicates for each combination, which we describe in Table 1.

Experiment 2: Testing for group formation upon exposure to predator products

Experimental tube cultures

We tested whether the addition of predator products led to algae forming groups. In the combination of *C. sorokiniana* with *Ochromonas* spp., we used nine 50-mL falcon tubes for the control without predator products, and nine 50-mL falcon tubes for the treatment where we added predator products. In each tube we placed 4.04 mL of *C. sorokiniana* to either 0.96 mL of filtered PPY in the control or 0.96 mL filtered liquid from a culture of *Ochromonas* in the treatment. The filter we used in both cases had a pore diameter of 0.22 μm. We kept the tube caps loose to allow for oxygenation and randomized all 18 tubes on a tube rack in an incubator at 20°C with a light/dark cycle of 16:8 hours using fluorescent illumination.

We obtained samples at five time points after adding the predator products: 1, 24, 48, 72, and 96 hours. At each time point, we tilted the falcon tubes five times, and transferred 200 μL of each culture into a 96-well plate. We minimized any possible bias in the collection of our data by shaking the cultures and then taking samples. We took one image from the centre of each well using a VisiCam digital camera under the inverted microscope at 20× magnification, and quantified the proportion of cells in groups. We followed the same experimental procedure for the remaining eight combinations, except for variation in the concentrations of algae and putative predator products, which we describe in Table 1. In the combinations with *D. magna*, instead of PPY, we added 0.96 mL of filtered Bolds Basal media to the algae in the control set.

Table 1. Concentrations, total volume per tube or well, and number of independent replicates used in the experiments

	Experiment	Final concentration of algae (cells/mL)	Final concentration of putative predators (cells/mL, individuals/mL or predator products from *x* individuals/mL)	Total volume (mL) per tube, well or flask	Number of independent replicates
C. sorokiniana with *Ochromonas* spp.	1	2×10^6	1×10^5	20	30*
	2	1×10^6	2×10^4	5	9
	3	3×10^6	4×10^5	5	9
	4	3×10^6	3×10^4	1	42
C. vulgaris with *Ochromonas* spp.	1	1×10^5	6×10^5	20	9
	2	1×10^6	2×10^4	5	9
	3	2×10^5	3×10^5	5	9
	4	3×10^6	5×10^4	1	42
S. obliquus with *Ochromonas* spp.	1, 3	2×10^5	3×10^5	5	9
	2	1×10^6	2×10^4	5	9
	4	1×10^6	2×10^4	1	42
C. sorokiniana with *T. thermophila*	1	3×10^6	1×10^4	20	30*
	2	1×10^6	3×10^6	5	9
	3	3×10^5	4×10^5	5	9
	4	2×10^6	1×10^5	1	42
C. vulgaris with *T. thermophila*	1	2×10^6	2×10^4	20	30**
	2	1×10^6	3×10^6	5	9
	3	2×10^5	4×10^5	5	9
	4	4×10^6	$\times 10^4$	1	42
S. obliquus with *T. thermophila*	1	2×10^5	4×10^5	5	9
	2	1×10^6	3×10^6	5	9
	3	1×10^5	4×10^5	5	9
	4	5×10^6	1×10^5	1	42
C. sorokiniana with *D. magna*	1, 3	9×10^6	1	5	9
	2a	1×10^6	3	5	9
	2b	2×10^4	0.2[#]	50	9
	4	9×10^6	1	1	3
C. vulgaris with *D. magna*	1, 3	5×10^6	1	5	9
	2a	1×10^6	3	5	9
	2b	2×10^4	0.2[#]	50	9
	4	5×10^6	1	1	3
S. obliquus with *D. magna*	1, 3	2×10^6	1	5	9
	2a	3×10^3	3	5	9
	2b	8×10^3	0.2[#]	50	9
	4	5×10^6	1	1	3

Notes: Experiment 1: testing for group formation and size change upon exposure to predators. Experiment 2: testing for group formation upon exposure to predator products. Experiment 3: testing for predation by measuring algal density. Experiment 4: testing for predation by observing ingestion. In Experiment 3, for the combinations of *S. obliquus* with *Ochromonas* spp. and *S. obliquus* with *T. thermophila*, we placed 4.04 mL of algae in the tubes with an additional 0. 96 mL of PPY in the control set and 0. 96 mL of the putative predator in the treatment set. Also in the same experiment, for the combinations with *Daphnia* (G–I), we placed 5 mL of algae in all tubes with an additional five *Daphnia* in each tube of the treatment set.

* $n_{1h} = 3, n_{24h} = 9, n_{48h} = 9, n_{72h} = 9$; ** $n_{1h} = 6, n_{24h} = 9, n_{48h} = 9, n_{72h} = 6$; [#]40 adult *Daphnia* in 200 mL of filtered *Daphnia* water.

We use the term 'predator products' to refer to anything present in the predator culture that can pass through a 0.22-μm filter. The filtered medium could contain products released from the predators, or even intracellular products released from fractured/dead predator cells.

Experimental flask cultures

We used the same methodology in all experiments to test the effect of predator products on group formation. However, in a previous study using *Scenedesmus* with *Daphnia*, Lampert *et al.* (1994) found that predator products did influence group formation. Consequently, we repeated the three combinations with *Daphnia*, following Lampert and colleagues' methodology. We transferred 200 mL of filtered Bolds Basal media and 200 mL of filtered water from the 1-litre culture jar of *D. magna* into two separate 250-mL Erlenmeyer flasks. In the latter, we added 40 adult *Daphnia*. We kept both flasks in an incubator for 24 hours at 22°C with a light/dark cycle of 16:8 hours using fluorescent illumination. We then added 2 mL of filtered liquid from the flask containing the filtered Bolds Basal media to 3 mL *S. obliquus* and 45 mL Bolds Basal media, in nine 250-mL Erlenmeyer flasks, for the treatment without the predator products. In the treatment with the predator products, we added 2 mL of filtered liquid from the flask containing the *Daphnia* to 3 mL *S. obliquus* and 45 mL Bolds Basal media, in nine 250-mL Erlenmeyer flasks. In all cases we used a filter with a pore diameter of 0.22 μm. We randomized all 18 flasks on a shaker at 280 rpm in an incubator at 22°C with a light/dark cycle of 16:8 hours using fluorescent illumination.

After 1, 24, 48, 72, and 96 hours, we transferred a sample of 200 μL from each of these flasks to a 96-well plate and took a photo from each well under the inverted microscope at 20× magnification. We performed image analysis using Image J software (Cell Counter plugin) and measured the proportion of cells in groups.

We followed the same methodology in the combinations of *C. sorokiniana* with *D. magna* and *C. vulgaris* with *D. magna*, apart from the algal concentrations used, which we describe in Table 1.

Experiment 3: Testing for predation by measuring algal density

We tested whether the addition of a predator had a significant impact on the algal populations. In the combination of *C. sorokiniana* with *Ochromonas* spp., we used nine 50-mL falcon tubes for the control without *Ochromonas,* and nine 50-mL falcon tubes for the treatment where we added the *Ochromonas* predator. In each tube we placed 4.04 mL of *C. sorokiniana* to either 0.96 mL of PPY in the control or 0.96 mL of *Ochromonas* spp. in the predator treatment. We incubated the tubes at 20°C with a light/dark cycle of 16:8 hours using fluorescent illumination and kept the tube caps loose to allow for oxygenation.

We obtained random samples at two time points: 0 hours, just before adding the putative predator, and 24 hours, after adding the putative predator. At each time point, we tilted the falcon tubes five times and transferred 200 μL of each culture into a 96-well plate. We took images with a VisiCam digital camera under the inverted microscope at 20× magnification. From these images, we counted the total number of algae and converted to $\log_{10}$ cells $\cdot$ mL^{-1}. We divided the algal density at 24 hours by the density at 0 hours to determine the relative change in algal density. We followed the same procedure for the rest of the predator–prey

combinations, with the concentrations, total volume used per tube, and number of independent replicates described in Table 1.

Experiment 4: Testing for predation by observing ingestion

Protists

We tested whether the protists ingested the algae by observing the protists' behaviour. For the *C. sorokiniana* with *Ochromonas* spp. combination, we added 980 μL of *C. sorokiniana* and 20 μL of *Ochromonas* spp. to each of 42 wells. We incubated the 24-well plates at 20°C with a light/dark cycle of 16:8 hours using fluorescent illumination. We observed the protists at seven time points: 1, 24, 48, 72, 96, 120, and 144 hours. At each time point we observed six independent wells. We followed one protist per well for 1 minute under an inverted microscope at 20× magnification to detect any ingesting activity towards unicells. Over the seven time points, we observed 42 protists in total. We took videos manually with a digital camera (Canon PowerShot A2600). We performed the same experiment for *C. vulgaris* with *Ochromonas* spp., *S. obliquus* with *Ochromonas* spp., *C. sorokiniana* with *T. thermophila*, *C. vulgaris* with *T. thermophila*, and *S. obliquus* with *T. thermophila*. The concentrations used are listed in Table 1.

Daphnia

We tested whether *Daphnia* ingested the algae by observing the colour of *Daphnia*'s gut in the presence of algae. We transferred 1 mL of *C. sorokiniana*, 1 mL of *C. vulgaris*, 1 mL of *S. obliquus*, and 1 mL of Bolds Basal media as a control, into four separate wells on a 24-well plate. We added one *Daphnia* to each of the four treatments, and replicated each treatment three times. We incubated the plate at 20°C with a light/dark cycle of 16 : 8 hours using fluorescent illumination. After 24 hours, we removed 900 μL from each well in order to minimize movement of the *Daphnia*, and took images of *Daphnia*'s gut with a digital camera (Canon Powershot A2600) under an inverted microscope at 4× magnification. The concentrations of algae that we used are listed in Table 1.

Statistical analysis

We carried out all analyses in R v. 3.2.3 (R Development Core Team, 2015). In the experiments where all data points were from different cultures and hence statistically independent (Experiment 1: *C. sorokiniana* with *Ochromonas* spp., *C. sorokiniana* with *T. thermophila*, *C. vulgaris* with *T. thermophila*), we estimated the overall difference in the proportion of cells in groups between the treatments with and without a predator, using a generalized linear model ('glm' package) with quasibinomial errors, to account for overdispersion of the data; for the mean group size we used a generalized linear model with Gaussian errors.

In the experiments where our data were repeated measurements from the same cultures (Experiment 1: *C. vulgaris* with *Ochromonas* spp., *S. obliquus* with *Ochromonas* spp., *S. obliquus* with *T. thermophila*, *C. sorokiniana* with *D. magna*, *C. vulgaris* with *D. magna*, *S. obliquus* with *D. magna*; Experiment 2: all algal–predator combinations), we compared the proportion of cells in groups across time between the treatments with and without a predator by fitting a generalized mixed-effects model with Penalized Quasi-Likelihood

('glmmPQL' package) using quasibinomial errors; for the mean size we used the same generalized mixed-effects model (glmmPQL), but with Gaussian errors. We then performed a Wald test on the overall effect of predator treatment to estimate *P*-values. We treated the interaction between predator treatment and time (Treatment × Time) as a fixed effect, and the repeated measurements as random effects (1 | Subject).

RESULTS

Do algae form groups in response to predators?

In all nine combinations, we observed significant group formation in response to the presence of the predator (Fig. 2; see figure legend for statistics). In most combinations, the proportion of cells in groups began to increase 24 hours after addition of the putative predator (Figs. 2A–H). In the combination *S. obliquus* with *D. magna*, the response appeared to be much faster, with the proportion of cells in groups going from only 14 ± 2%

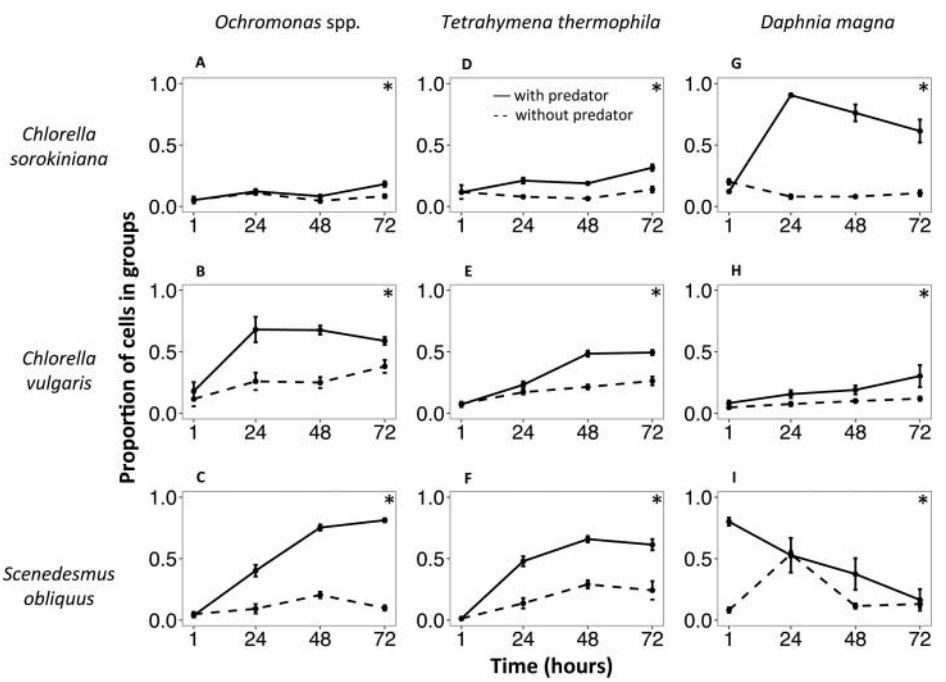

Fig. 2. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In all nine combinations, the proportion of cells in groups was higher in the presence of the putative predator [A: generalized linear model (glm), $F = 11.93$, $P < 0.01$; B: generalized mixed-effects model using Penalized Quasi-Likelihood (glmmPQL), $P < 0.0001$; C: glmmPQL, $P < 0.0001$; D: glm, $F = 77.23$, $P < 0.0001$; E: glm, $F = 66.047$, $P < 0.0001$; F: glmmPQL, $P < 0.0001$; G: glmmPQL, $P < 0.0001$; H: glmmPQL, $P < 0.001$; I: glmmPQL, $P < 0.0001$]. The term 'predator' in the legend refers to a putative predator. The asterisk represents a significant difference ($P < 0.05$) in the overall main effect of treatment across time. Error bars represent standard error of the mean.

of cells before the *Daphnia* were added, to 80 ± 3% of cells just one hour after the *Daphnia* were added (Fig. 2I; glm at 'time point 1 hour', $F = 202.7$, $P < 0.0001$).

Does group size change?

Group size increased in the presence of the predator in seven combinations (Figs. 3C–I and 4C–I). For example, in the *S. obliquus* with *D. magna* combination, the mean group size increased from 3.3 ± 0.1 before the *Daphnia* were added, to 9.7 ± 0.8 just one hour after adding the *Daphnia* (Fig. 3I; glm at 'time point 1 hour', $F = 51.1$, $P < 0.0001$). The two combinations in which group size did not increase were *C. sorokiniana* with *Ochromonas* and *C. vulgaris* with *Ochromonas.* The different algal species formed different types of groups (Fig. 5). In *Chlorella*, groups were irregularly shaped. In *Scenedesmus*, groups varied in size and morphology – for example, we observed four-celled (Fig. 5E) and eight-celled groups, where cells were attached sideways, as well as chain-like groups, where cells were attached by their ends (Fig. 5C, D).

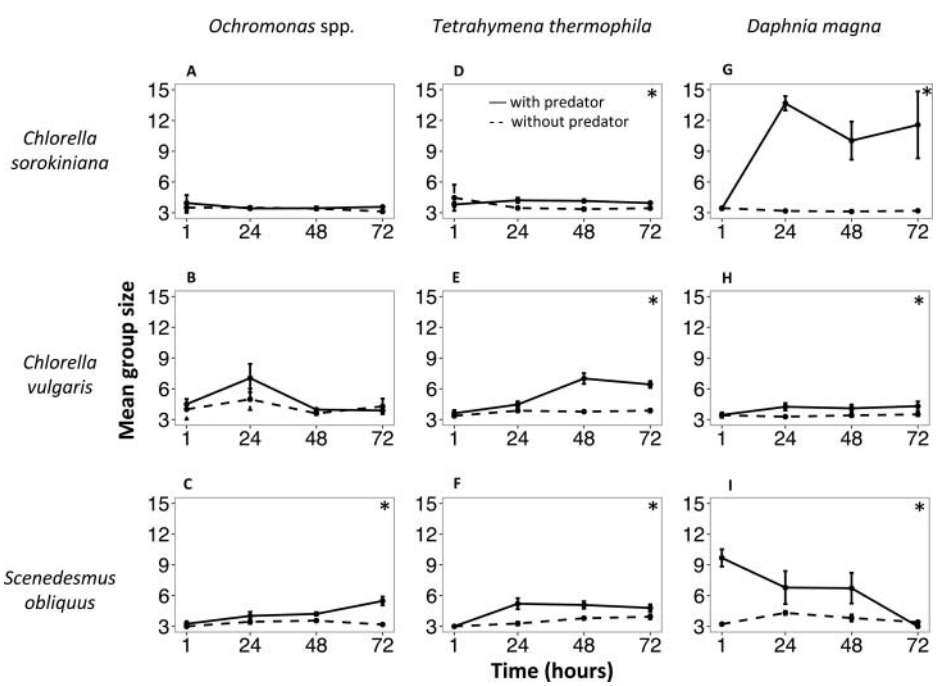

Fig. 3. Mean group size plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In seven combinations, the mean algal group size was higher in the presence of the putative predator (C: glmmPQL, $P < 0.0001$; D: glm, $F = 11.48$, $P < 0.01$; E: glm, $F = 63.39$, $P < 0.0001$; F: glmmPQL, $P < 0.0001$; G: glmmPQL, $P < 0.0001$; H: glmmPQL, $P = 0.011$; I: glmmPQL, $P < 0.0001$). In two combinations, the mean group size did not increase in the presence of the putative predator (A: glm, $F = 2.07$, $P = 0.156$; B: glmmPQL, $P = 0.3$). The asterisk represents a significant difference ($P < 0.05$) in the overall main effect of treatment across time. Error bars represent standard error of the mean.

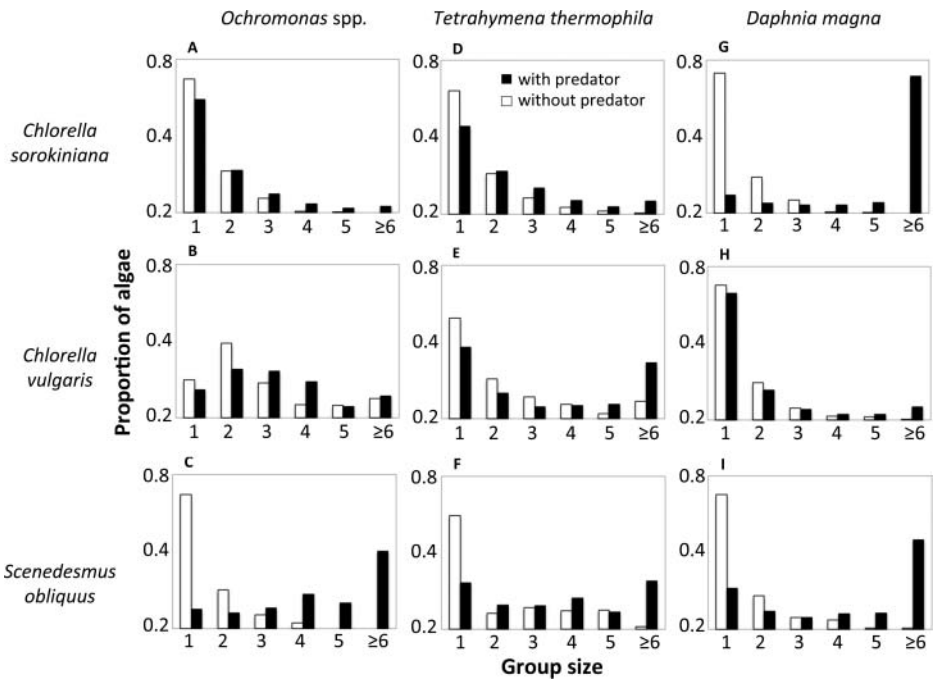

Fig. 4. Distribution of group sizes in the presence (solid bars) and absence (open bars) of the putative predator after 72 hours (A–F) and 48 hours (G–I). Group sizes '1' and '2' refer to a unicell and a paired cell, respectively.

Do algae form groups in response to predator products?

In three combinations – *C. vulgaris* with *T. thermophila* (Fig. 6a.E), *C. sorokiniana* with *D. magna* (Fig. 6a.G), and *S. obliquus* with *D. magna* (Fig. 6b.I) – algae formed groups in response to predator products. In the experiments conducted in tube cultures, we observed group formation in two combinations (Figs. 6a.E, G). We repeated the combinations with *Daphnia* using Lampert and colleagues' (1994) methodology, and observed that in *S. obliquus* with *D. magna*, the algae formed groups in response to *Daphnia* products (Fig. 6b.I).

Do the predators impact algal density?

The addition of the potentially predatory species led to a decrease in algal density in five of nine combinations (Figs. 7A, B, G–I). The four combinations in which we did not observe a decrease in algal density were: *S. obliquus* with *Ochromonas* spp. (Fig. 7C), *C. sorokiniana* with *T. thermophila* (Fig. 7D), *C. vulgaris* with *T. thermophila* (Fig. 7E), and *S. obliquus* with *T. thermophila* (Fig. 7F).

Predator behavioural observations

In relation to *Ochromonas*, 9.5% of the time (4/42 observations) we observed *Ochromonas* capturing *C. sorokiniana* (Fig. 8A); 7.1% of the time (3/42) capturing *C. vulgaris* (Fig. 8B); and none of the time (0/42) *Ochromonas* exhibiting any ingesting activity towards

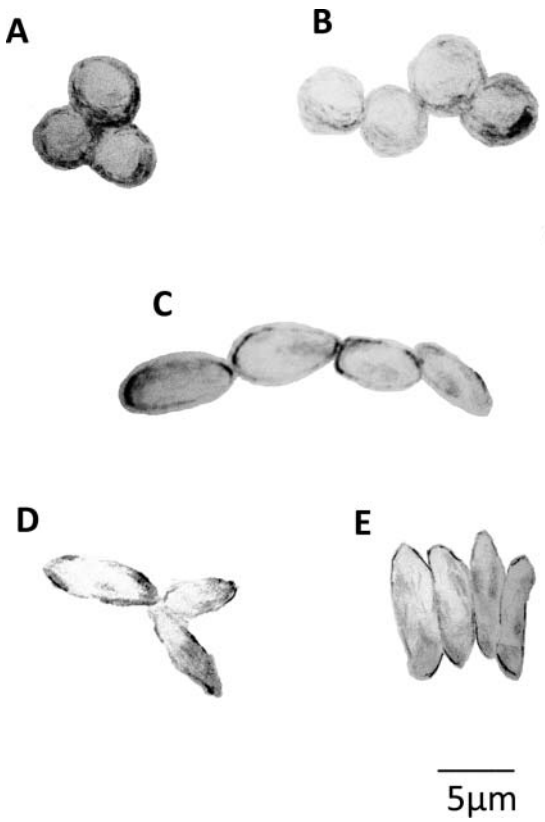

Fig. 5. Characteristic algal group types: (A) three-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (B) four-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (D) three-celled group observed in *S. obliquus* cultures; (C, E) four-celled groups seen in *S. obliquus* cultures.

S. obliquus. Regarding *Tetrahymena*, 7.1% of the time (3/42) *Tetrahymena* ingested *C. sorokiniana* (Fig. 8D); 80.9% of the time (34/42) *C. vulgaris* algae were visible inside *Tetrahymena* (Fig. 8E); and 2.3% of the time (1/42) *S. obliquus* was seen inside *Tetrahymena* (Fig. 8F). Considering *Daphnia*, 100% of the time (3/3) *Daphnia*'s gut was green in the presence of the algae (Figs. 8G–I).

DISCUSSION

Overall, in the nine 'algal–predator' combinations that we tested: (1) the presence of live predators led to a higher proportion of cells going into groups in all nine combinations (Fig. 2), and groups being composed of larger numbers of cells in seven combinations (Fig. 3); (2) the presence of predator products induced algal group formation in three combinations (Fig. 6); (3) the presence of predators resulted in a decrease in algal density in five combinations (Fig. 7), and behavioural observations consistent with predation, in eight combinations (Fig. 8).

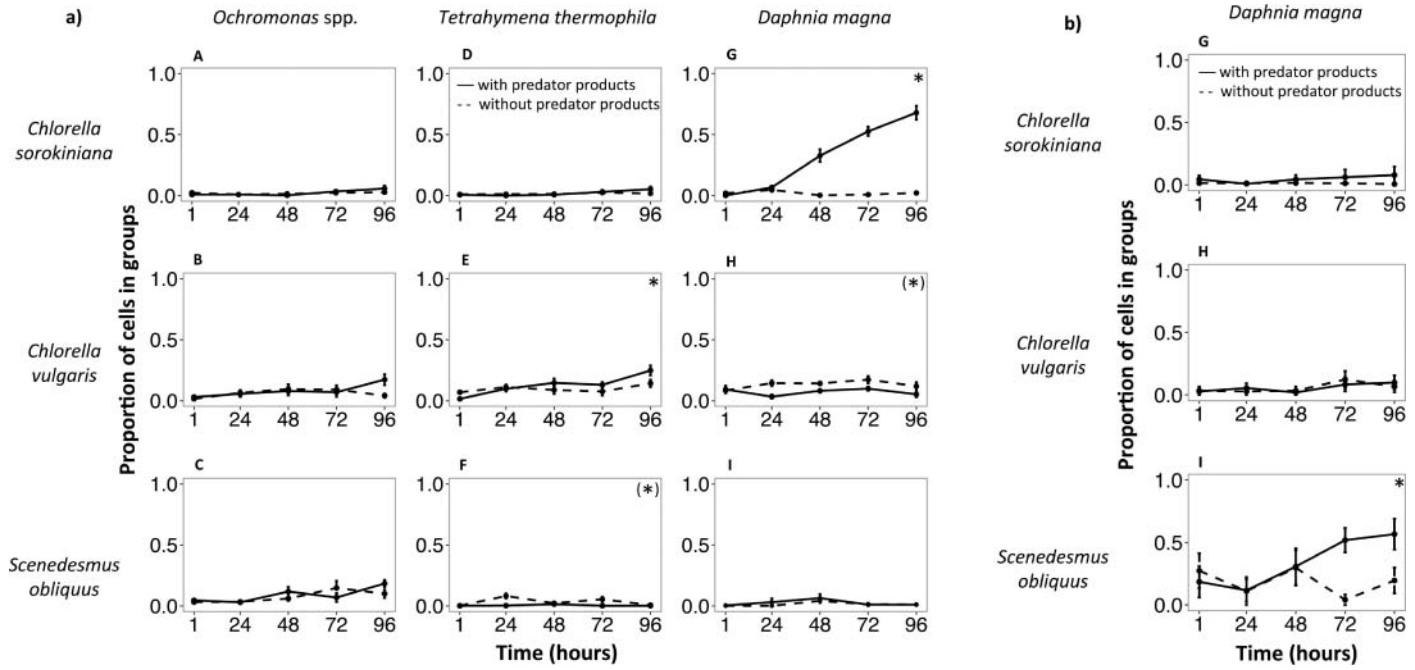

Fig. 6. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of predator products. (a) Experiment in tube cultures. In two combinations, the proportion of cells in groups was higher in the presence of the predator products (glmmPQL, E: $P < 0.01$; G: $P < 0.0001$). In five combinations, the proportion of cells in groups did not increase (glmmPQL, A: $P = 0.052$; B: $P = 0.059$; C: $P = 0.063$; D: $P = 0.3$; I: $P = 0.76$). And in two combinations, the proportion of cells decreased in the presence of the putative predator (glmmPQL, F: $P < 0.01$; H: $P < 0.001$). (b) Experiment in flask cultures. In the case of *S. obliquus* with *D. magna*, the proportion of cells in groups was higher in the presence of the predator products (glmmPQL, I: $P < 0.01$). In the other two combinations, the proportion of cells in groups did not increase (glmmPQL, G: $P = 0.46$; H: $P = 0.92$). The asterisk represents a significant difference ($P < 0.05$) in the overall main effect of treatment across time. Error bars represent standard error of the mean.

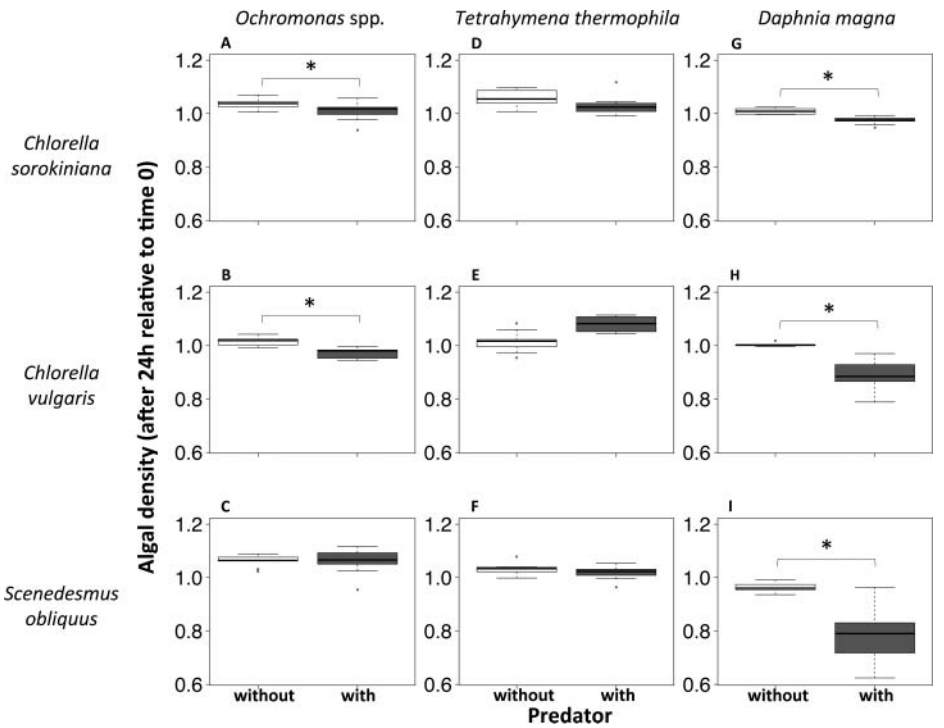

Fig. 7. Algal density (after 24 hours relative to time 0) in the presence (grey) and absence (white) of the putative predator. In five combinations, the algal density decreased in the presence of the putative predator (two-sample *t*-test, A: d.f. = 16, $P = 0.041$; B: d.f. = 16, $P < 0.0001$; G: d.f. = 16, $P < 0.0001$; H: d.f. = 16, $P < 0.0001$; I: d.f. = 16, $P < 0.001$). In four combinations, the algal density did not decrease in the presence of the putative predator (two-sample *t*-test, C: d.f. = 16, $P = 0.987$; D: d.f. = 16, $P = 0.120$; E: d.f. = 16, $P = 0.075$; F: d.f. = 16, $P = 0.313$). The asterisk represents a significant difference ($P < 0.05$) in the overall main effect of treatment across time.

Response to predators

In all nine combinations, the addition of predators led to a higher proportion of cells in groups (Fig. 2), and in seven combinations, predators led to the formation of larger groups (Figs. 3 and 4). In certain combinations, such as *C. sorokiniana* with *D. magna* and *S. obliquus* with *D. magna*, this group formation was so extreme that groups were visible with the naked eye (Fig. 9). That predation induced group formation in all combinations suggests that group formation can be a relatively general response to predators. However, it has previously been found that the alga *Scenedesmus acutus* does not form groups in the presence of the predators *Chydorus sphaericus*, *Cyclops agilis* or *Cypridopsis vidua* (Van Donk *et al.*, 1999), indicating that the response to predation is not a completely general response by all related species.

Previous studies have shown group formation in three combinations that were the same or very similar to the nine that we examined. Von Elert and Franck (1999) have shown that *S. obliquus* forms groups in the presence of *D. magna*, but they did not measure the proportion of cells in groups. Fisher *et al.* (2016) showed that the proportion of *C. vulgaris*

cells in groups increased in the presence of *T. thermophila*, in 24-well plates. Boraas *et al.* (1998) found that *C. vulgaris* formed groups upon predation by *Ochromonas vallescia*. Our study differs from that of Boraas *et al.* (1998) in that they used *C. vulgaris* CCAP 211/8A with *O. vallescia* in chemostat cultures, and did not statistically analyse group formation, whereas we used *C. vulgaris* CCAP 211/11B with *Ochromonas* spp. in tube cultures. Our data suggest that the algae may produce different group sizes in response to different predators,

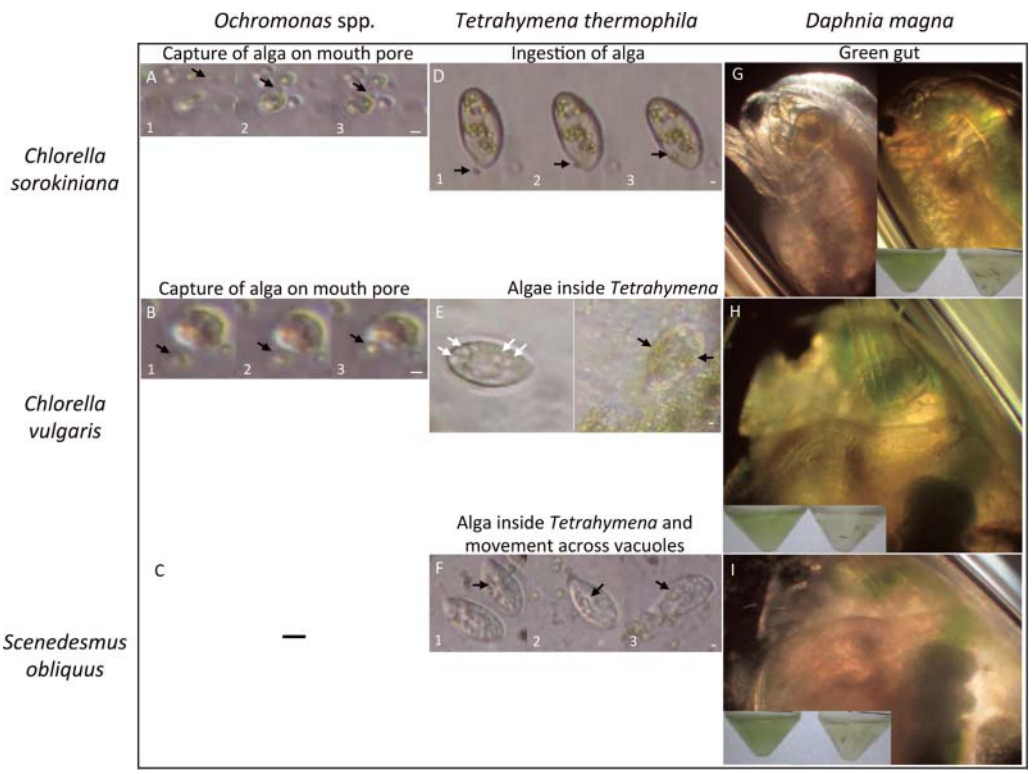

Fig. 8

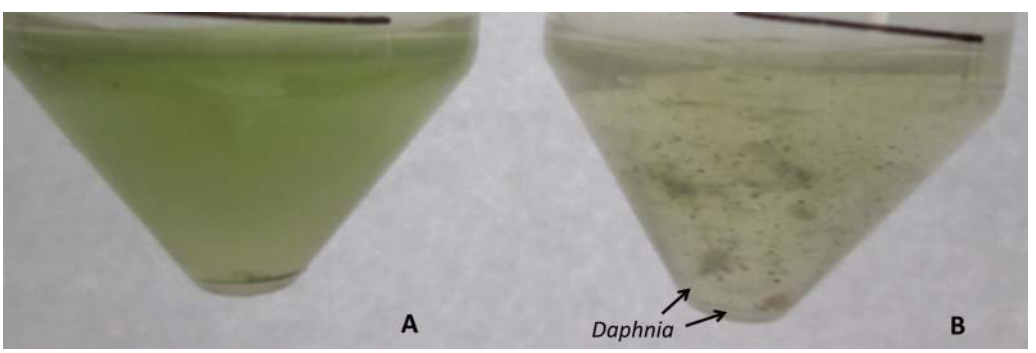

Fig. 9

possibly because optimal group size depends upon the type of predator (Figs. 2 and 3). However, our study was not designed to test this hypothesis, as the different species were studied at different times, and so future work will be required to formally test this.

Is group formation a behavioural response of the algae?

We tested whether algae form groups facultatively, in response to cues of predator presence, by exposing algae to filtered liquid from a culture of live predators. We found that in three combinations – *C. vulgaris* with *T. thermophila* (Fig. 6a.E), *C. sorokiniana* with *D. magna* (Fig. 6a.G), and *S. obliquus* with *D. magna* (Fig. 6b.I) – the algae responded to predator products by forming groups. In the other six combinations, we cannot exclude the possibility of group formation being a behavioural response, since the group-inducing signal may be the actual presence of predators, or cues from algal fed predators.

Group formation in response to predator products has been previously observed in the case of *C. vulgaris* with *T. thermophila* in 24-well plates (Fisher *et al.*, 2016) and in *S. acutus* (later classified as *S. obliquus*; www.ukncc.co.uk) with *D. magna* in flask cultures. However, in our experiment with tube cultures we did not observe group formation in *S. obliquus* in response to *Daphnia* (Fig. 6a.I). This discrepancy may have been due to differences in methodology, which differed in many respects (see Methods). Therefore, we repeated our three combinations with *Daphnia* using Lampert and colleagues' (1994) methodology; when we did this, we found group formation in *S. obliquus* in response to *Daphnia* products (Fig. 6b.I), confirming Lampert and colleagues' finding, but no group formation in *C. sorokiniana*

Fig. 8. Direct observations of protists' feeding behaviour and *Daphnia*'s gut. Images A, B, and D–F are video snapshots. Black arrows show algae. (A) Capture of unicellular *C. sorokiniana* by *Ochromonas*. *Chlorella sorokiniana* rotates upon contact with *Ochromonas*'s mouth pore and flagella (A2); it then stops rotating and remains in contact with *Ochromonas* (A3). (B) Capture of unicellular *C. vulgaris* by *Ochromonas*. *Chlorella vulgaris* rotates upon contact with *Ochromonas*'s mouth pore and flagella (B1, B2); it then stops rotating and remains in contact with *Ochromonas* (B3). (C) – indicates no observed feeding behaviour towards the alga. (D) Ingestion of unicellular *C. sorokiniana* by *T. thermophila*. *Chlorella sorokiniana* passes through *Tetrahymena*'s mouth pore (D2, D3). (E) Left image: *Tetrahymena* cultured in Bolds Basal media without algae for 24 hours. White arrows show empty vacuoles, which are indicative of starvation (Nakajima *et al.*, 2009). Right image: *Tetrahymena* cultured with *C. vulgaris* for 24 hours. Green algae are visible inside *Tetrahymena*. (F) Unicellular *S. obliquus* inside *T. thermophila* and passage from one vacuole to another: At first, *S. obliquus* is enclosed in the frontal vacuole of *T. thermophila* (F1). Next, the frontal vacuole and an adjacent vacuole join and form a larger vacuole (F2). *Scenedesmus obliquus* is initially positioned in the centre and is then gradually positioned in the lower part of the large vacuole (F2). The large vacuole splits into two separate vacuoles, and *S. obliquus* is enclosed in the second vacuole (F3). Scale bars on images A, B, and D–F are 5 μm. (G) Left image: gut coloration of *D. magna* after 24 hours with no added algae. Right image: noticeable green gut 24 hours after adding *C. sorokiniana*. (H) Green gut 24 hours after adding *C. vulgaris*. (I) Green gut 24 hours after adding *S. obliquus*. After 72 hours, green algal cultures (bottom left tube: without *Daphnia*) had become almost transparent due to grazing by *D. magna* (bottom right tube: with *Daphnia*).

Fig. 9. Group formation in *C. sorokiniana* upon predation by *D. magna*. Cultures of *C. sorokiniana*, incubated 72 hours in the absence (A) and presence of *D. magna* (B). Groups of *C. sorokiniana* are visible in the liquid culture (B) as well as two *Daphnia* (arrows).

(Fig. 6b.G). This emphasizes that methodological differences between experiments can produce contrasting results. Previous studies have identified a compound, 8-methylnonyl sulphate, that is produced by *D. magna* and induces group formation in *Scenedesmus* (Yasumoto *et al.*, 2005; Uchida *et al.*, 2008).

Our study raises a number of questions to do with how groups form. Groups can form by the association of the daughter cells with the parent cell after cell division, or by the aggregation of cells. The mechanism matters, because cooperation is more likely to be favoured with parent–daughter cell associations, as this leads to a higher relatedness (Fisher *et al.*, 2013). Previous studies have shown that *S. acutus* (Lürling and Van Donk, 2000) and *C. vulgaris* (Boraas *et al.*, 1998) form groups through such parent–daughter cell associations. Although we did not directly test how groups form, our observation that *S. obliquus* forms groups within 1 hour (Fig. 2I), before the cells have divided, indicates that *S. obliquus* may be forming groups by aggregation. Another issue is that group formation may be facultative, or a fixed genetic response. Although we did not test between these alternatives, the speed with which groups formed, and the fact that it could be driven by predator products (see, for example, Fig. S2: 3034Appendix.pdf), suggest a facultative response, with groups being formed under certain conditions.

Predation

We found that the presence of predators led to a decrease in algal density, consistent with significant predation, in five combinations (Figs. 7A, B, G–I). We did not observe decreased algal density in four combinations: *S. obliquus* with *Ochromonas* spp. (Fig. 7C), *C. sorokiniana* with *T. thermophila* (Fig. 7D), *C. vulgaris* with *T. thermophila* (Fig. 7E), and *S. obliquus* with *T. thermophila* (Fig. 7F). Fisher *et al.* (2016) did not observe a decrease in the density of *C. vulgaris* upon predation by *T. thermophila* either. In these four cases (Figs. 7C–F), *Ochromonas* spp. and *T. thermophila* were either poor predators, or algal group formation was so successful that it prevented the algae being grazed upon. Nakajima *et al.* (2013) suggested that aggregation of *C. vulgaris* reduces the rate of ingestion by *T. thermophila*.

Our behavioural observations (Figs. 8A, B, D–I) suggested that in eight combinations the predators were eating the algae. Specifically, *Ochromonas* spp. captured *C. sorokiniana* (Fig. 8A) and *C. vulgaris* (Fig. 8B) on its mouth pore. The algae rotated as soon as they reached the flagella of *Ochromonas* and then stopped rotating. Although this observation may at first not directly imply ingestion, Boraas *et al.* (1992) reported that as soon as 50% of the *C. vulgaris* cell is enveloped by *Ochromonas*, the *C. vulgaris* cell stops rotating and then the cell is 'drawn into the body of *O. vallescia*'. This suggests that our observation may be a preliminary step before ingestion. In the cases of *T. thermophila* with *C. sorokiniana* (Fig. 8D), *C. vulgaris* (Fig. 8E), and *S. obliquus* (Fig. 8F), we clearly saw ingestion of the algae and presence of the alga inside *T. thermophila*, respectively. *Chlorella vulgaris* algae have been previously observed inside vacuoles of *T. thermophila* (Nakajima *et al.*, 2009).

In all the combinations with *D. magna* (Fig. 8G–I), we observed a green coloration of *Daphnia*'s gut. This has previously been seen in the combinations of *D. magna* with *C. vulgaris* (Ryther, 1954) and *S. obliquus* (Lürling and Verschoor, 2003), but not with *C. sorokiniana*. In the combinations with *Daphnia*, the benefit of group formation may be to increase survival during gut passage, rather than to decrease predation. For example, *Daphnia* induced the non-gelatinous unicellular *Sphaerocystis schroeteri* to form gelatinous groups,

and these groups passed through *Daphnia*'s gut, where they gained nutrients from the remains of edible algae and *Daphnia*'s metabolites. The algae then emerged intact from *Daphnia*'s gut, due to their protective gelatinous sheath (Porter, 1976; Kampe *et al.*, 2007). In another experiment, *D. magna* ingested the algae *C. vulgaris* and then green masses of undigested *C. vulgaris* were excreted from *D. magna*'s gut (Ryther, 1954).

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DATA ACCESSIBILITY

The experimental data for this study are freely available on Dryad:
http://datadryad.org/resource/doi:10.5061/dryad.78nq4.

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