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The costs and benefits of multicellular group formation in algae*

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The first step in the evolution of complex multicellular organisms involves single cells forming a cooperative group. Consequently, to understand multicellularity, we need to understand the costs and benefits associated with multicellular group formation. We found that in the facultatively multicellular algae *Chlorella sorokiniana*: (1) the presence of the flagellate *Ochromonas danica* or the crustacean *Daphnia magna* leads to the formation of multicellular groups; (2) the formation of multicellular groups reduces predation by *O. danica*, but not by the larger predator *D. magna*; (3) under conditions of relatively low light intensity, where competition for light is greater, multicellular groups grow slower than single cells; (4) in the absence of live predators, the proportion of cells in multicellular groups decreases at a rate that does not vary with light intensity. These results can explain why, in cases such as this algae species, multicellular group formation is facultative, in response to the presence of predators.

KEY WORDS: ***Chlorella*, cooperation, major evolutionary transitions, multicellularity, predation.**

A blue whale is a cooperative group of about 100 quadrillion cells (Zhang et al. 2005). In order for such a large and complex multicellular organism to have evolved, the single-celled ancestor of this species must have joined together to form a cooperative multicellular group (Maynard Smith and Szathmary 1995; Grosberg and Strathmann 2007; Michod 2007; Bourke 2011; Claessen et al. 2014; West et al. 2015). This poses an evolutionary problem, because grouping together is likely to have costs, such as increased competition for resources, which would have to be outweighed by any benefits of group formation. The costs and benefits of multicellular group formation cannot be examined in complex multicellular organisms, like the blue whale, because they cannot complete their life cycle as single cells (they are obligately multicellular). In addition, such species also exhibit division of labor between different cells, a subsequent elaboration, which only arose after the formation of multicellular groups. Consequently, if we want to understand the factors that favored the initial evolution of multicellular groups, we need to examine species that can exist both as single cells and multicellular groups (facultatively multicellular), and before division of labor has evolved. Possible benefits to forming multicellular groups include that multicellular groups are better able to defend against predators, disperse, or forage for food and resources (Strassmann et al. 2000; Grosberg and Strathmann 2007; Bourke 2011; Koschwanez et al. 2013; Claessen et al. 2014; Smith et al. 2014; Biernaskie and West 2015).

Facultatively multicellular algae offer excellent opportunities for measuring the benefits and costs of multicellular group formation. Many species of green algae live as single cells, until certain ecological conditions, such as the presence of predators, cause them to form multicellular groups (Van Donk et al. 2011; Fisher et al. 2016; Kapsetaki et al. 2016). Previous work examining the costs and benefits of group formation in algae has produced mixed results. Some studies have found that group formation provides a defense against predators, but others have not (Hessen and Van Donk 1993; Lampert et al. 1994; Lürling and Van Donk 1996; Lürling 1999). One possible reason for this variation could be that forming groups provides a defense against relatively small predators, but not against relatively large predators that can consume groups.

A possible explanation for why group formation is only facultative is that it could incur costs, and so would only be favored when the benefits outweigh the costs. Most studies have failed to find a cost of group formation, in terms of increased competition for resources (Riegman et al. 1992; Peperzak 1993; Boraas et al. 1998; Lurling and Van Donk 2000; Jakobsen and Tang 2002; Lurling and Beekman 2006). However, costs and benefits will

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depend upon environmental conditions, and the conditions that influence group formation could have confounding influences. For example, a failure to find a cost of group formation, in terms of decreased growth rates, may reflect experiments being carried out in benign environments, where resources were not limiting (Lurling and Van Donk 2000; Jakobsen and Tang 2002; Lurling and Beekman 2006).

We examined the costs and benefits of multicellular group formation in the algae *Chlorella sorokiniana*. This species forms multicellular groups facultatively, in response to the presence of a number of predators, including the flagellate *Ochromonas* spp., the ciliate *Tetrahymena thermophila*, and the crustacean *Daphnia magna* (Kapsetaki et al. 2016). We have previously shown that group formation can also be induced by the addition of products from a culture of one of these predators, *D. magna*, in the absence of actual predators (Kapsetaki et al. 2016). We exploited this as an experimental tool, to manipulate whether cells are in groups, independently of other factors.

We had three specific aims. First, we tested whether multicellular group formation led to reduced predation by the flagellate predator *Ochromonas danica* or by the crustacean predator *D. magna*. We used *O. danica* and *D. magna* because: (1) both species predate *C. sorokiniana*, and (2) *O. danica* is approximately 100 times smaller than *D. magna*, allowing us to examine a small predator that is a similar size as the algae, and a large predator that can be much larger than groups of algae (Lürling et al. 1997; Boraas et al. 1998; Kapsetaki et al. 2016). Second, we tested whether, in the absence of predators, multicellular group formation led to reduced growth. We examined for this potential cost of group formation under conditions of both high and low light availability, because the growth cost of forming groups could depend upon environmental conditions (Lürling and Van Donk 2000; Jakobsen and Tang 2002; Lurling and Beekman 2006).

Third, we tested whether multicellular groups broke up, and returned to single cells, in the absence of predators, and whether this varied between high and low light availability. If group formation is costly, then we would expect group breakup to be favored when the benefit (predator avoidance) was not there, and especially under conditions where resources are limiting, and hence the costs of group formation are higher (Dehning and Tilzer 1989; Boraas et al. 1998; Verschoor et al. 2009). In addition to our specific experimental aims, we also examined the generality of our results, with a meta-analysis on data compiled from the literature.

Methods

STRAINS

We maintained the algae *C. sorokiniana* (nonaxenic from the Culture Collection of Algae and Protozoa; CCAP; Scotland; UK; strain number 211/8K) in Bolds Basal media at 20°C at a light:dark cycle of 16:8 h fluorescent illumination (LMS incubator—Model 280; Kent; UK). To eliminate bacteria from the cultures, we added 500 μg mL^{-1} of the antibiotic rifampicin to 1 mL samples of *C. sorokiniana*, and diluted the samples 1:300 after 24 h in Bolds Basal media (CCAP; Scotland; UK; Kapsetaki et al. 2016; Kapsetaki et al. 2017). No bacteria were visible under an inverted microscope after rifampicin treatment. In addition, before each experiment, we took culture samples, and observed them under an inverted microscope at $\times$20 and $\times$40 magnification, to test for possible bacterial contamination. In the richer media, for instance Proteose Peptone Yeast extract (PPY; CCAP; Scotland; UK), contamination was more frequent. In cases where there was contamination, we did not carry out the experiment. Instead, we started a new culture from the stock culture, treated this new culture with rifampicin, and started the experiment afresh. We maintained the cultures in 1-L Erlenmeyer flasks shaking at 220 rpm, at 20°C and a light:dark cycle of 16:8 h fluorescent illumination, before using the cultures in experiments.

As putative predators, we used *O. danica* (axenic from the National Center for Marine Algae and Microbiota; Maine; USA; strain number CCMP 3279) cultured in 2.5 mL PPY media diluted in 7.5 mL dH_2O, and *D. magna* (Sciento, Manchester, UK), which we fed 5 mL *Scenedesmus obliquus* (10^6 cells mL^{-1}) every 4–5 days. A number of previous studies have shown that *Ochromonas* and *Daphnia* species can predate algae (Ryther 1954; Daley et al. 1973; Boraas et al. 1992; Lürling and Verschoor 2003). We maintained the *O. danica* culture in a 50 mL Falcon tube and the *D. magna* in 500-mL jars and at 20°C and a light:dark cycle of 16:8 h fluorescent illumination. Before using *O. danica* in experiments, we reverse filtered the culture with Bolds Basal media to remove *O. danica* from the nutrient-rich PPY media and thus minimize the possibility of PPY-induced group formation in *C. sorokiniana* (Fisher et al. 2016; Kapsetaki et al. 2016). The filter we used in all experiments had a pore diameter of 0.22 μm. The resulting final concentration of *O. danica* that we used in experiments was 1.5×10^6 cells mL^{-1}.

To induce group formation in the algae *C. sorokiniana*, we used filtered liquid from the predator *D. magna* (Kapsetaki et al. 2017). *D. magna* is a much larger predator, whose extracts have a much larger influence on group formation, and hence allow us greater experimental power (Kapsetaki et al. 2016; Kapsetaki et al. 2017).

EXPERIMENT 1: IS THERE A FITNESS BENEFIT OF BEING IN A GROUP?

Ochromonas predator

Our previous work with *C. sorokiniana* used a different *Ochromonas species*, *Ochromonas* spp. Consequently, our first aim was to test whether the algae *C. sorokiniana* form groups in response to live *O. danica* and/or supernatant from *O. danica.* We

added 1 mL of *C. sorokiniana* (10^6 cells mL^{-1}) with 0.75 mL filtered Bolds media to either: (1) 0.75 mL of *O. danica*; (2) 0.75 mL filtered liquid from the culture of *O. danica*; (3) 0.75 mL filtered Bolds media as a negative control of group formation; or (4) 0.75 mL filtered liquid from the culture of *D. magna* (final concentration of three individuals per milliliter) as a positive control of group formation in 50-mL Falcon tubes (15 replicates per treatment). We randomized the tubes on tube racks incubated at 20°C and a light:dark cycle of 16:8 h fluorescent illumination. We kept the tube caps loose to allow oxygenation. We collected samples at 0 and 24 h by tilting each tube five times to adequately mix the cultures, and transferring 50 μL from each culture into a 96-well plate. We minimized sampling bias by obtaining an image from a random area of each well with a VisiCam digital camera under an inverted microscope (VWR, Model XDS-3; Optica; Landsberg am Lech; Germany) at ×20 magnification. In these two-dimensional images, we quantified the proportion of cells in groups (number of algal cells in groups/total number of algal cells). We performed image analysis using Image J software (Cell Counter plugin; National Institutes of Health; Laboratory for Optical and Computational Instrumentation; University of Wisconsin; USA). We define a group as ≥3 cells in contact with each other, but found the same qualitative results when analyzing mean group size. In several cases, the exact number of cells in three-dimensional groups, especially in large groups, was difficult to determine from the two-dimensional images, as many cells were "hidden in the background." For consistency, we counted what we saw in the two-dimensional images. We counted paired cells as single cells in all the experiments. We counted cell numbers based on the images obtained from the microscope rather than OD measurements, because we noticed that the light from the spectrophotometer interferes with multicellular groups and so does not provide an accurate measure of total cells when there are groups in a culture.

Second, we tested whether *O. danica* is a predator of *C. sorokiniana* and whether groups of *C. sorokiniana* have a higher fitness relative to single cells when exposed to *O. danica*. We added 4.04 mL of *C. sorokiniana* (10^6 cells mL^{-1}) to either: (1) 0.96 mL of filtered liquid from the culture of *D. magna* (final concentration of three individuals per milliliter) ($n = 10$ "multicellular" cultures); or (2) 0.96 mL of filtered Bolds media in a 50-mL Falcon tube ($n = 10$ "unicellular" cultures). We maintained the 20 tubes at 20°C at 16:8 h light:dark cycle fluorescent illumination for 96 h. We mixed replicates of the same treatments resulting in two cultures, and diluted both cultures to 40 algal cells per field of view at ×20 magnification. We then created four experimental treatments in a 2 × 2 factorial design, with multicellular or unicellular cultures, and with or without the addition of *O. danica* predators. We did this by combining into 50-mL Falcon tubes 0.75 mL of filtered Bolds media with: (1) 1 mL of the multicellular culture, and 0.75 mL of *O. danica*; (2) 1 mL of the multicellular culture, and 0.75 mL filtered liquid from the culture of *O. danica*; (3) 1 mL of the unicellular culture, and 0.75 mL *O. danica*; or (4) 1 mL of the unicellular culture, and 0.75 mL filtered liquid from the culture of *O. danica*. We replicated each treatment 30 times. We randomized the tubes on tube racks, keeping the tube caps loose to allow oxygenation, and incubated at 20°C and a light:dark cycle of 16:8 h. After adding the predator treatments, we collected samples at 0 and 3 h, sampling as described above, to measure the total number of algal cells and the proportion of cells in groups.

A potential complication with this experiment is that the addition of *O. danica* predators induces multicellular group formation in the algae, which could reduce the difference between our treatments in the proportion of algae in multicellular groups. To avoid this, we quantified predation just 3 h after adding predators—live *O. danica* induces group formation in *C. sorokiniana* after 24 h, but not after 3 h (Kapsetaki et al. 2016). We also used a relatively low density of algae in this experiment as mentioned above (40 algal cells per field of view at ×20 magnification) because prior experiments showed that a related small predator *Ochromonas* spp. cannot eat a very large number of cells especially in such a short amount of time (3 h; Kapsetaki et al. 2016).

Daphnia predator

We performed three experiments to test whether group formation is beneficial in terms of avoiding predation by *D. magna*. We examined whether cultures of unicellular algae experience a larger decrease in density than multicellular cultures after exposure to: (1) mixed sizes of *D. magna*; (2) small-, medium-, or large-sized *D. magna*; and (3) whether cells that were in multicellular groups were less likely to be predated by *D. magna* in cultures that contained both multicellular groups and unicells. This combination of experiments allowed us to test if multicellular group formation helped reduce predation, if this benefit only occurred with certain size *D. magna*, or if *D. magna* preferentially fed on unicells/smaller groups.

First, we examined whether unicellular cultures decrease in density more than multicellular cultures after adding mixed sizes of *D. magna*. We added 4 mL of *C. sorokiniana* (10^6 cells mL^{-1}) to either: (1) 1 mL of filtered liquid from the *D. magna* culture (final concentration of three individuals per milliliter) ($n = 30$ "multicellular" cultures); or (2) 1 mL of filtered Bolds media in a 50-mL Falcon tube ($n = 30$ "unicellular" cultures). We kept tubes at 20°C at 16:8 h light:dark cycle fluorescent illumination for 96 h. We mixed replicates of the same treatments resulting in two cultures, and diluted both cultures to 10^6 cells mL^{-1}. We then created four experimental treatments, with multicellular or unicellular cultures, and with or without the addition of *D. magna* predators of mixed sizes. Specifically, we combined into 50-mL

Falcon tubes: (1) 4.5 mL of the multicellular culture, and 0.5 mL of filtered liquid from the culture of *D. magna*; (2) 4.5 mL of the multicellular culture, and 0.5 mL with 15 individuals *D. magna* of various sizes; (3) 4.5 mL of the unicellular culture, and 0.5 mL of filtered liquid from the culture of *D. magna*; or (4) 4.5 mL of the unicellular culture, and 0.5 mL with 15 individuals *D. magna* of various sizes. We replicated each treatment 18 times. We randomized tubes on tube racks, keeping the tube caps loose allowing gas exchange, and incubated at 20°C and a light:dark cycle of 16:8 h. After adding the predator treatments, we collected samples at 0 and 12 h, sampling as described above, to measure the total number of algal cells and the proportion of cells in groups.

Second, we tested whether groups of *C. sorokiniana* have a benefit in terms of avoiding predation by small, medium, or large *D. magna*. We carried out this experiment because any benefit of avoiding predation could vary with predator size, and so could have been masked by using *D. magna* of variable sizes. We followed the same protocol as above, but instead of the treatments where we added *D. magna* of various sizes, we had three separate treatments where we added small (<2 mm), medium (2–3 mm), or large (>3 mm) *D. magna*.

Third, we tested whether multicellular groups of algae were less likely to be predated by *D. magna* in cultures that contained both multicellular groups and unicells. We carried out this experiment in case the benefit of avoiding predation was only realized when there was a range of group sizes available, for example, if *D. magna* preferentially feed on unicells. We differentiated between cells that were from multicellular or unicellular cultures by marking them with different fluorescent dyes. We prepared unicellular and multicellular cultures by either adding filtered Bolds media or filtered *D. magna* supernatant to the algae *C. sorokiniana*, then separated each culture in two separate treatments, dyed each treatment with either a green or violet fluorescent dye, mixed together the differently dyed multicellular and unicellular cultures at a 1:1 ratio, before adding the *D. magna*.

We added 2.5 mL of *C. sorokiniana* (10^6 cells mL^{-1}) in 50-mL Falcon tubes to either 2.5 mL of filtered Bolds media, or 2.5 mL filtered liquid from the *D. magna* culture (final concentration of three individuals per milliliter) (six replicates per treatment). We kept the Falcon tube caps loose to allow for gas exchange and randomized tubes on a rack in the incubator at 20°C with a light/dark cycle of 16:8 h using fluorescent illumination.

After 48 h, we mixed together the tubes from the same treatments and followed the protocol for fluorescently dyeing cells as described in Kapsetaki et al. (2017). We centrifuged the cultures at 100 × *g* for 10 min and resuspended the pellet in CD-CHO Medium (Gibco, Carlsbad, CA; Thermo Fisher Scientific; Paisley; UK). We then split each culture in equal volumes and added the fluorescent dye CellTracker™ Green BODIPY (final concentration 20 μM; Thermo Fisher Scientific; Paisley; UK) to one culture, and CellTracker™ Violet BMQC (final concentration 20 μM; Thermo Fisher Scientific; Paisley; UK) to the other culture. We diluted stock dyes in 10 mM DMSO. We covered the four cultures with aluminum foil, left them shaking at 170 rpm overnight at room temperature, then centrifuged both cultures at 100 × *g* for 10 min, and resuspended in Bolds media to remove the dyes.

We diluted the cultures to 10^6 cells mL^{-1}, and mixed together the multicellular green-dyed culture with the unicellular violet-dyed culture, and the multicellular violet-dyed culture with the unicellular green-dyed culture at a 1:1 volume ratio.

We then combined into 50-mL Falcon tubes: (1) 1 mL of the multicellular green:unicellular violet culture and three medium-sized *D. magna*; (2) 1 mL of the multicellular violet:unicellular green culture and three medium-sized *D. magna.* We used 18 replicates for each treatment. We randomized tubes on tube racks, keeping the tube caps loose to allow oxygenation, and incubated at 20°C under fluorescent illumination. After adding the predators, we collected 20 μL samples at 0 and 3 h, constructed fluid tunnel slides (Kapsetaki et al. 2017), and imaged samples under a Zeiss Axio Zoom V16 fluorescence stereoscope (Carl Zeiss, Oberkochen, Germany) at ×100 magnification. We took three images per replicate and measured the number of green and violet cells. We averaged these three experimental replicates to a single data point, leading to 18 independent data points per treatment.

EXPERIMENT 2: IS THERE A FITNESS COST OF BEING IN A GROUP?

We examined whether cells growing in groups have lower growth rates than cells growing as unicells, and whether this was influenced by light availability. We manipulated light availability by wrapping some tubes uniformly with aluminum foil up to the 30-mL indication (Fig. 4A), which reduced light and subsequent algal growth (see section "Results"). We transferred 4.04 mL of *C. sorokiniana* (10^6 cells mL^{-1}) to either: (1) 0.96 mL of filtered Bolds media in Falcon tubes; (2) 0.96 mL of filtered Bolds media in Falcon tubes wrapped with aluminum foil (see Fig. 4A); (3) 0.96 mL of filtered liquid from the culture of *D. magna* (final concentration of three individuals per milliliter) in Falcon tubes; or (4) 0.96 mL of filtered liquid from the culture *of D. magna* in Falcon tubes wrapped with aluminum foil. We used five independent replicates for each treatment. We randomized tubes on tube racks and incubated them at 20°C at a light:dark cycle of 16:8 h fluorescent illumination, keeping the tube caps loose to allow for oxygenation. After adding the predator treatments, we collected samples at 0 and 96 h, using the abovementioned sampling protocol, and measured the total number of algal cells and the proportion of cells in groups.

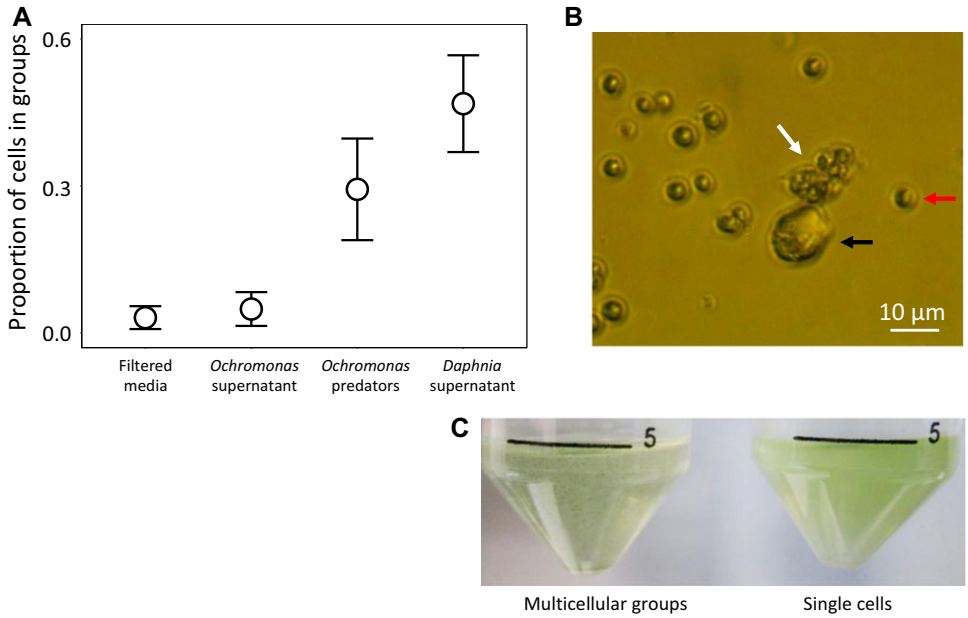

Figure 1. **Group formation in the algae *C. sorokiniana*. (A) The addition of both live *O. danica* predators and *D. magna* supernatant, but not the addition of *O. danica* supernatant, led to an increase in the proportion of cells in multicellular groups. These results are in comparison to our control treatment, where we added filtered media. Error bars show 95% confidence intervals (CI; 15 independent replicates per treatment). (B) Single cells of the algae *C. sorokiniana* (red arrow) are smaller than their *O. danica* predator (black arrow). Multicellular groups of *C. sorokiniana* (white arrow) can be similar in size or larger than their *O. danica* predator. (C) Group formation in the algae *C. sorokiniana* is visible to the naked eye. The two tubes show *C. sorokiniana* incubated for 96 h in the presence (left) and absence of *D. magna* supernatant (right). Groups of *C. sorokiniana* are visible on the left where multicellular group formation had been induced.**

Next, following our observation of lower algal growth in multicellular cultures than unicellular cultures grown in darkness, we examined whether this decline was due to competition of cells for light, supporting the hypothesis that multicellular group formation is costly, or simply due to multicellular groups sinking to the bottom of tubes, thus cells inhibiting other cells from exposure to light essential for their growth. We performed the same experiment as above, but instead of the dark treatments, we used "dark with hole" treatments where we removed aluminum foil from the bottom of the tubes creating a hole through which light could pass (Fig. 5A). We used 15 independent replicates per treatment.

EXPERIMENT 3: DO GROUPS REVERT TO UNICELLULARITY FASTER IN THE DARK?

In this experiment, we tested whether the rate at which groups of *C. sorokiniana* break up, from multicellular groups to single cells (unicells), is influenced by the light level. We placed 4.04 mL of *C. sorokiniana* to either 0.96 mL of filtered Bolds media ($n = 18$), or 0.96 mL filtered liquid from the *D. magna* culture (final concentration of three individuals per milliliter) ($n = 18$) in 50-mL Falcon tubes. We kept the tube caps loose to allow for oxygenation and randomized all 36 tubes on tube racks in the incubator at 20°C with a light:dark cycle of 16:8 h using fluorescent illumination. On Day 4, we covered 50% of the tubes that had been treated with *D. magna* supernatant and 50% of the tubes that had been treated with filtered Bolds media, in aluminum foil (Fig. 4A). We left the remaining tubes without aluminum foil. After adding the *D. magna* supernatant, we obtained samples on Day 0 (0 h), 1 (24 h), 4, 7, 10, 13, 16, 19, and 22, using the sampling protocol described above, and quantified the proportion of cells in groups. In the beginning of the experiment, the proportion of cells in groups did not differ between the four treatments (anova-glm, $F_{3,32} = 0.07$, $P = 0.97$).

STATISTICAL ANALYSIS

We performed most of our statistical analyses with generalized linear models, in the statistical package R (version 3.2.3; "glm" package; Crawley 2012; R Core Team 2015). When analyzing the proportion of cells in groups, we assumed binomial errors, with the family "quasibinomial" to correct for data overdispersion. We carried out our analyses by step-wise deletion to the minimal adequate model. The only experimental analysis that we did not carry out with a generalized linear model was when examining the effect of *Daphnia* size (large, small, and medium) on algal density where we carried out a regression analysis using a linear model

and when examining how groups break up in experiment 3. In the latter, because our data were repeated measures over time we fitted a generalized mixed-effects model with Penalized Quasi-Likelihood ("glmmPQL" package) using quasibinomial errors. We treated the interaction between the two treatments ("light with *Daphnia* supernatant," "dark with *Daphnia* supernatant") and time as a fixed effect and the repeated measurements across time as random effects. We set Day 4 as the starting time point in our analysis for comparing how groups break up over time in light versus darkness, because that is when the tubes were separated into light and dark conditions, and set time as a second-degree orthogonal polynomial to allow for curvature in the fit.

Results

EXPERIMENT 1: IS THERE A FITNESS BENEFIT OF BEING IN A GROUP?

Consistent with our previous results, we found that the algae *C. sorokiniana* form multicellular groups in response to the addition of live *O. danica* or supernatant from a culture of *D. magna* ($F_{1,58} = 123.14$, $P < 0.0001$; Fig. 1; Kapsetaki et al. 2016), but not in response to supernatant from a culture of *O. danica* ($F_{1,57} = 1.50$, $P = 0.22$; Fig. 1A). We exploited this by using *D. magna* supernatant as a way to experimentally manipulate the extent to which cultures of *C. sorokiniana* were in multicellular groups.

Ochromonas predator

We found that: (1) the presence of *Ochromonas* relative to just *Ochromonas* supernatant significantly reduced the number of algae in unicellular cultures indicating that *Ochromonas* is a predator of *C. sorokiniana* (Fig. 2B); (2) the formation of multicellular groups in the algae *C. sorokiniana* reduced predation by *O. danica* (Fig. 2). Three hours after adding *O. danica* predators, the number of algal cells was significantly lower in the experimental replicates containing unicellular algae relative to those where we had induced multicellular group formation prior to adding the predators (interaction term: $F_{1,116} = 8.19$, $P = 0.004$). We observed cells of *C. sorokiniana* inside *O. danica*, consistent with *O. danica* predating *C. sorokiniana* (Fig. S4). Furthermore, the algal density in our treatment where we induced multicellular groups prior to adding predators was not significantly different from our two controls, where we added *O. danica* supernatant rather than live *O. danica* to either unicellular or multicellular groups ($F_{2,116} = 0.10$, $P = 0.89$; Fig. 2B).

Daphnia predator

In contrast, in all three of our experiments with *D. magna*, formation of multicellular groups by the algae *C. sorokiniana* did not reduce predation (Fig. 3). First, the number of algal cells in unicellular and multicellular cultures did not decrease at different rates after adding mixed sizes of *D. magna* (interaction term: $F_{1,68} = 0.41$, $P = 0.52$; Fig. 3A). Second, when we added different sizes of *D. magna*, the predation rate did not differ between unicellular and multicellular cultures (interaction term: $F_{3,136} = 1.92$, $P = 0.12$; Fig. 3B). The reduction in number of algae (predation) was greater with larger *D. magna* (Fig. 3B; linear model, adjusted $r^2 = 0.38$, $F_{1,106} = 68.71$, $P < 0.0001$). Third, in cultures containing a mixture of multicellular and unicellular algal cells, the

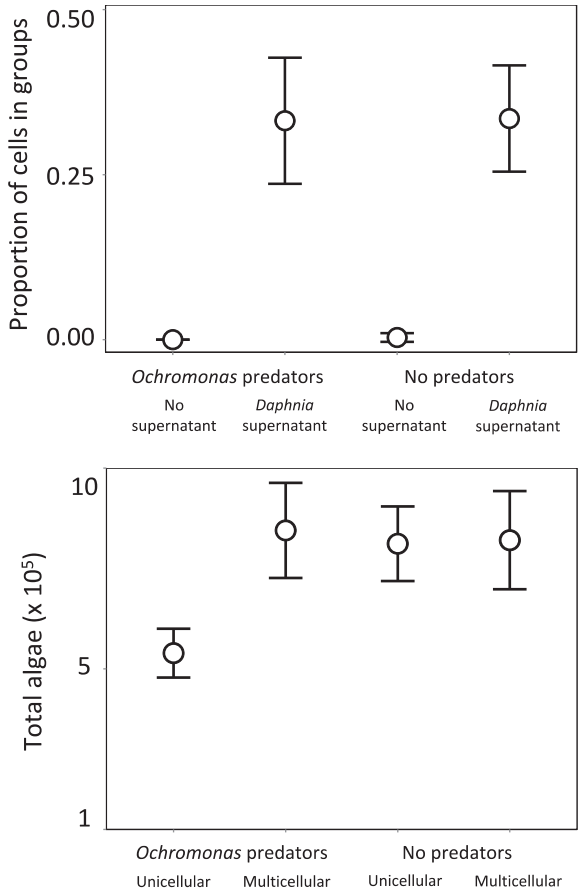

Figure 2. The benefit of multicellular group formation in the algae *C. sorokiniana* upon predation by *O. danica*. (A) The proportion of algal cells in multicellular groups was manipulated by adding *D. magna* supernatant. The addition of *D. magna* supernatant, prior to adding *O. danica* predators, led to a significantly higher proportion of cells in multicellular groups, as also shown in Figure 1. In contrast, the proportion of cells in multicellular groups did not change significantly in response to 3 h exposure to *O. danica* predators. (B) Multicellular group formation prevented predation by *O. danica*. Three hours after adding *O. danica* predators, the total number of algal cells was reduced in the cultures containing unicellular algae, but not in the cultures containing multicellular algae. These differences are compared to the control treatments where we did not add *O. danica* predators. In both plots, error bars indicate 95% CI (30 independent replicates per treatment).

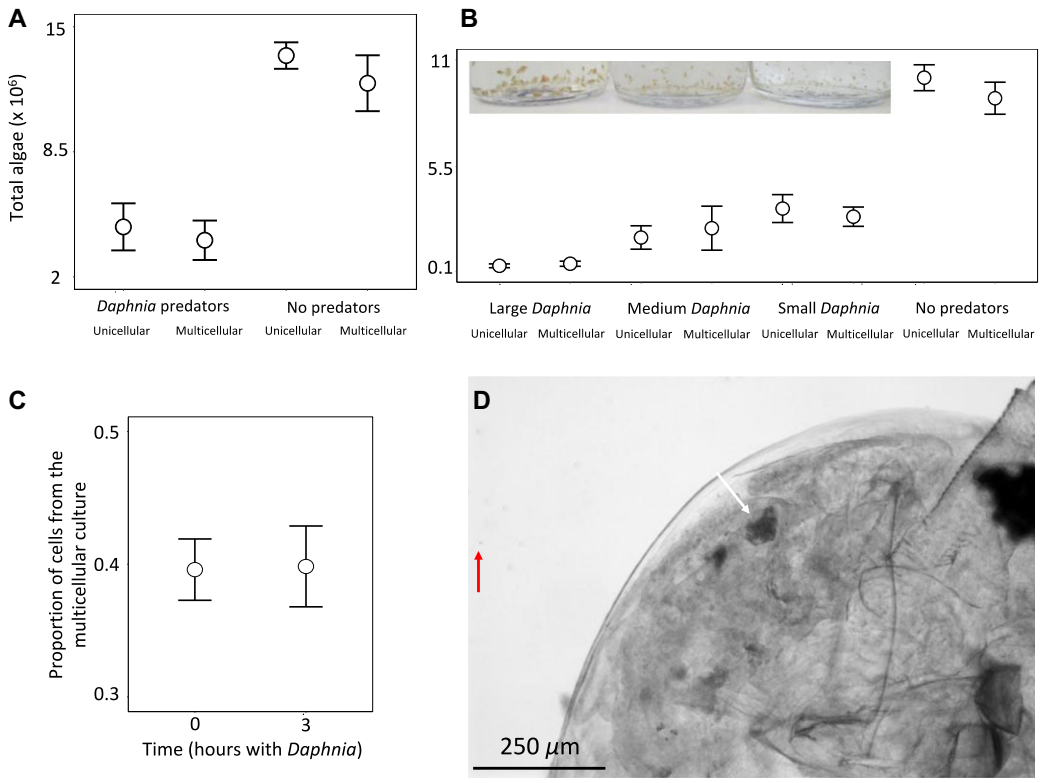

Figure 3. **Multicellular group formation does not reduce predation by *D. magna*. (A) The rate at which *D. magna* predators predated algae did not vary significantly dependent upon whether the algae were in multicellular groups. (B) Across different sized *D. magna* predators, the rate at which they predated algae did not vary significantly dependent upon whether the algae were in multicellular groups. Larger *D. magna* caused a larger decrease in algal density. (C) In mixed algal cultures, there was no significant difference in the rate at which *D. magna* predated multicellular groups versus unicells. Error bars in all plots (A–C) represent 95% CI (18 independent replicates per treatment). (D) A multicellular group (white arrow) of *C. sorokiniana* can be seen inside the *D. magna*, indicating ingestion. A single cell of *C. sorokiniana* (red arrow) outside the *D. magna* is shown for size comparison.**

proportion of cells originating from the multicellular culture did not change significantly after adding medium-sized *D. magna*, suggesting that multicellular groups were not differently predated than unicellular groups ($F_{1,70} = 0.25$, $P = 0.61$; Fig. 3C). These medium-sized *D. magna* decreased total algal density after 3 h ($F_{1,69} = 15.39$, $P = 0.0002$), indicating predation.

EXPERIMENT 2: IS THERE A FITNESS COST OF BEING IN A GROUP?

We examined the cost of multicellular group formation by comparing the growth rate of populations with different proportions of cells in multicellular groups under conditions of high resource availability (light) and low resource availability (dark). As in our benefit experiment, we manipulated the proportion of cells in multicellular groups by adding in *D. magna* supernatant (Fig. 4B; $F_{1,18} = 269.28$, $P < 0.0001$).

We found that whether populations were maintained under light or dark conditions did not influence the proportion of cells growing in multicellular groups (Fig. 4B; $F_{1,17} = 0.54$, $P = 0.46$; interaction term: $F_{1,16} = 1.93$, $P = 0.18$). Growing cells under relatively dark conditions led to reduced growth rate when algae were in multicellular groups, but not when they were growing as unicells (Fig 4C; $F_{1,16} = 12.56$, $P = 0.002$). This suggests that the costs of multicellular group formation are greater when light is more limiting.

An alternate hypothesis for our above result is that in the dark treatment, multicellular groups have higher sinking rates than single cells, sink to the bottom of the tube, and due to the density of algae in the precipitate, they receive less light and thus grow less than single cells in the dark. To distinguish between these two hypotheses, we repeated our experiment, but instead allowed light to pass through the bottom of tubes covered in aluminum foil ("dark with hole" treatments; Fig. 5A), thus enabling growth of any precipitate, which may have formed in the multicellular culture. We found that multicellular cultures also grew less than unicellular cultures in the "dark with hole" treatments, suggesting that multicellular cultures experienced greater competition (Fig. 5C; $F_{1,56} = 6.19$, $P = 0.01$). Algae formed larger precipitates in single-celled cultures than in multicellular cultures (Fig. S1), further supporting the hypothesis that the observed decrease in

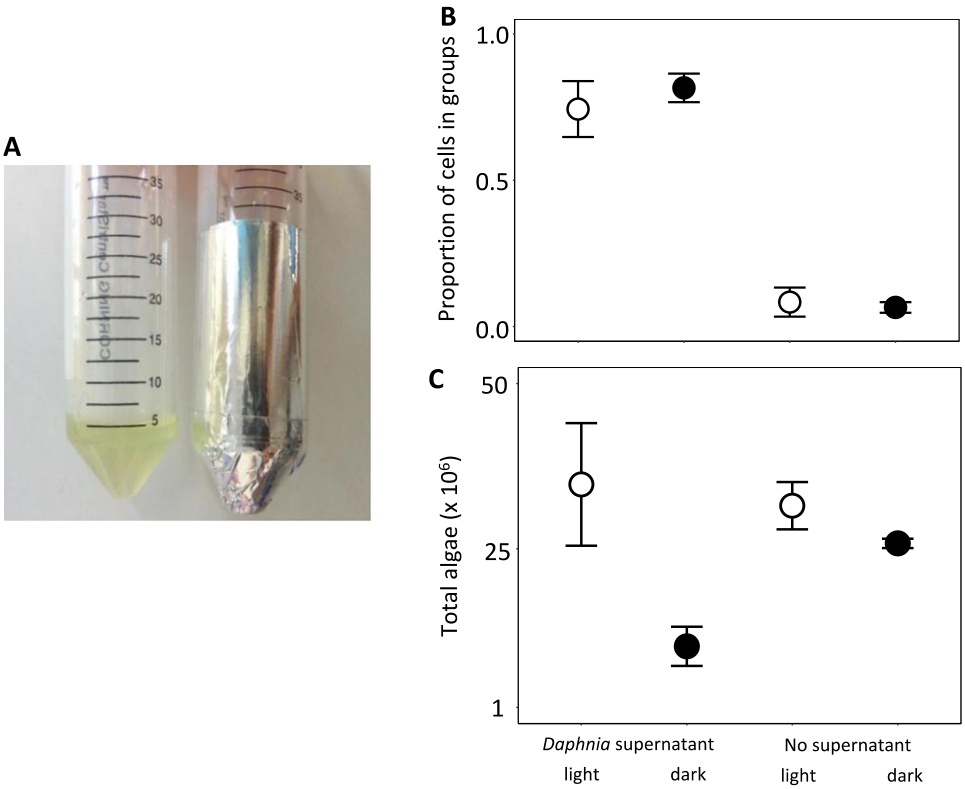

Figure 4. **The cost of group formation in the algae *C. sorokiniana*. (A) We used aluminum foil to reduce the light availability. The left tube is the "light" treatment and the right tube is the "dark" treatment. (B) The addition of *D. magna* supernatant led to a higher proportion of cells being in multicellular groups. Light levels did not influence the proportion of cells in multicellular groups. (C) Algae with more cells in multicellular groups grew to lower cell densities under dark, but not light, conditions. This suggests there is a cost of multicellular group formation, but only when light is limiting. In plots (B) and (C), error bars show 95% CI (five independent replicates per treatment).**

algal density (Fig. 4C) is due to multicellular groups competing for light, not due to sinking.

EXPERIMENT 3: DO GROUPS BREAK UP FASTER IN THE DARK?

Examining the treatments where we used extract of predator culture to induce multicellular group formation, the proportion of cells in groups significantly decreased over time (Fig. 6; glmmPQL, $P < 0.0001$). This decrease in the proportion of cells in groups could potentially be explained by either existing multicellular groups breaking up, or by the new cells being produced remaining predominantly unicellular (causing the absolute number of cells not in groups to increase over time). Although, the rate of growth was lower in the dark treatments (Fig. S3). In addition, examining the average size of multicellular groups, between Day 4 and Day 22 in the treatments with *Daphnia* supernatant, we found a significant decrease from 9 ± 1 ($\pm$95% CI) to 4 ± 1 in the light treatment (glmmPQL, $P = 0.001$), but no significant change in the dark treatment (Day 4: 8 ± 1, Day 22: 10 ± 3; glmmPQL, $P = 0.37$).

The rate at which the proportion of cells in groups decreased did not vary significantly depending upon whether cells were maintained in the dark or light (Fig. 6; from Day 4 onward, glmmPQL, $P = 0.08$; Day 4, proportion of cells in groups in the light and dark treatments with *Daphnia* supernatant $\pm$95% CI, respectively: 0.39 ± 0.07, 0.33 ± 0.08; on Day 22 the same comparison: 0.14 ± 0.05, 0.25 ± 0.09).

Discussion

We found that in the algae *C. sorokiniana*, the formation of multicellular groups can provide both a benefit and a cost. In particular, group formation (1) reduces predation by *O. danica* (Fig. 2B, Movie S1); (2) does not reduce predation by the larger predator *D. magna* (Fig. 3); and (3) leads to reduced algal growth under conditions of relatively low light intensity (Figs. 4C, 5). Over time, in the absence of predators, the proportion of cells in multicellular groups decreases, this decrease did not differ between algae grown in the dark or light (Fig. 6).

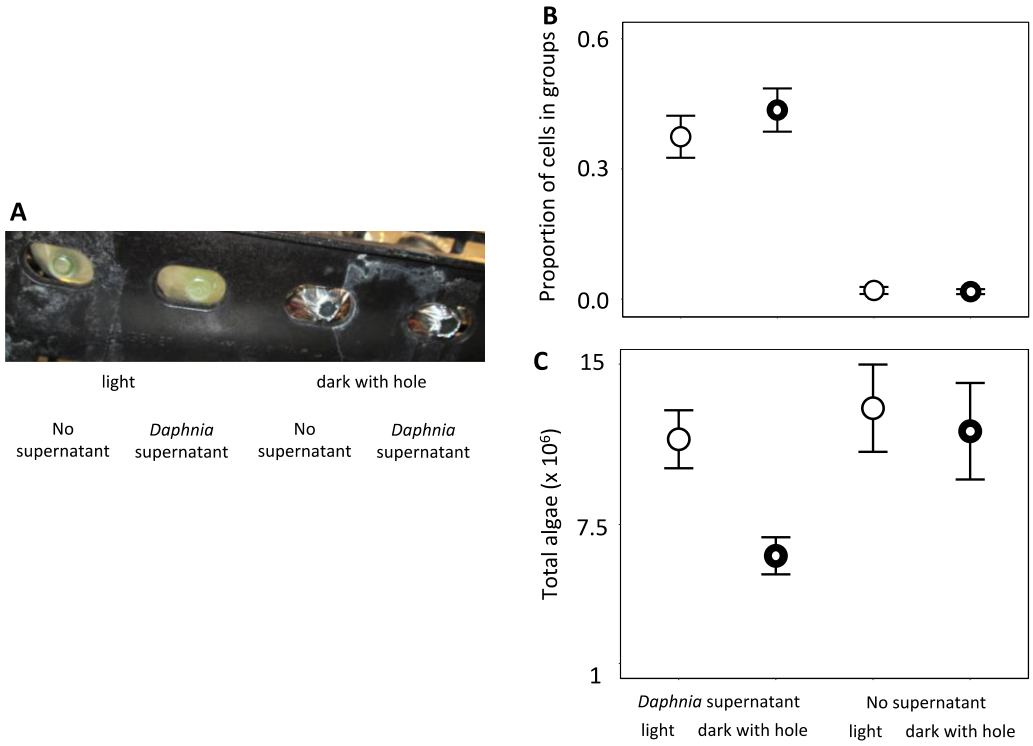

Figure 5. **The cost of group formation is not due to sinking. (A) The first two cultures from the left are the "light" treatments, and the remaining two are the "dark with hole" treatments. In the latter, light was allowed to pass from the bottom of tubes by creating a small hole in the aluminum foil. (B) The proportion of cells in groups was not influenced by light levels. (C) Multicellular cultures grew less than unicellular cultures in the "dark with hole" treatment, but not in the "light" treatment, indicating that this decreased growth is not caused by sinking, that is, cells restricting other cells access to light. Both plots show error bars of 95% CI (15 independent replicates per treatment).**

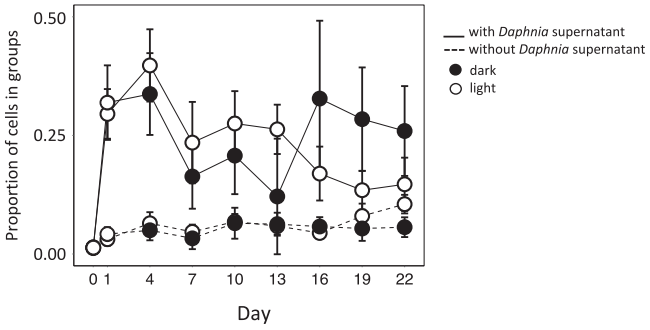

Figure 6. **Multicellular groups of the algae *C. sorokiniana* did not break up faster in the dark than in the light. At time point 0, we exposed 50% of the 36 replicate tubes of algae to *D. magna* supernatant (solid line) leading to an increase the proportion of cells in multicellular groups. On Day 4, we wrapped 50% of the tubes in aluminum foil, to produce a "dark" treatment. The proportion of cells in multicellular groups did not significantly vary depending upon whether they were kept in light or dark conditions. Error bars show 95% CI.**

BENEFITS OF MULTICELLULAR GROUP FORMATION

Previous studies examining the benefits of multicellular group formation in algae have produced mixed results (Hessen and Van Donk 1993; Lürling 1999; Herron et al. 2018). In *Scenedesmus acutus*, *S. obliquus*, and *Scenedesmus subspicatus*, group formation has been suggested to reduce predation by the predators *Brachionus calyciflorus* (Lürling 1999), *D. magna* (Hessen and Van Donk 1993), and *Daphnia cucullata* (Lürling and Van Donk 1996; Lürling et al. 1997), but not in *S. obliquus* upon exposure to the predators *D. magna* (Lürling et al. 1997) or *Daphnia pulex* (Lürling and Van Donk 1996). Multicellular groups might escape predation for a number of reasons, including being too large for predators to ingest, being better able to survive the predator's gut, or by sinking to a predatory free zone (Ryther 1954; Porter 1976; Dehning and Tilzer 1989; Sterner 1989; Kretzschmar et al. 1993; Kampe et al. 2007). It has also been suggested that several protists, including *Gyrodinium dominans*, *Ochromonas vallescia*, and *Euplotes* sp., are able to ingest single algal cells but cannot ingest groups of algae (Weisse and Scheffel-Möser 1990; Boraas et al. 1992; Boraas et al. 1998; Tang et al. 2001; Jakobsen and Tang 2002; Long et al. 2007).

We found that multicellular group formation reduces predation of the algae *C. sorokiniana* by a small predator (*O. danica*), but not by a large predator (*D. magna*) (Figs. 2B, 3A, 3D). We confirmed that our negative result with *D. magna* was not due to a

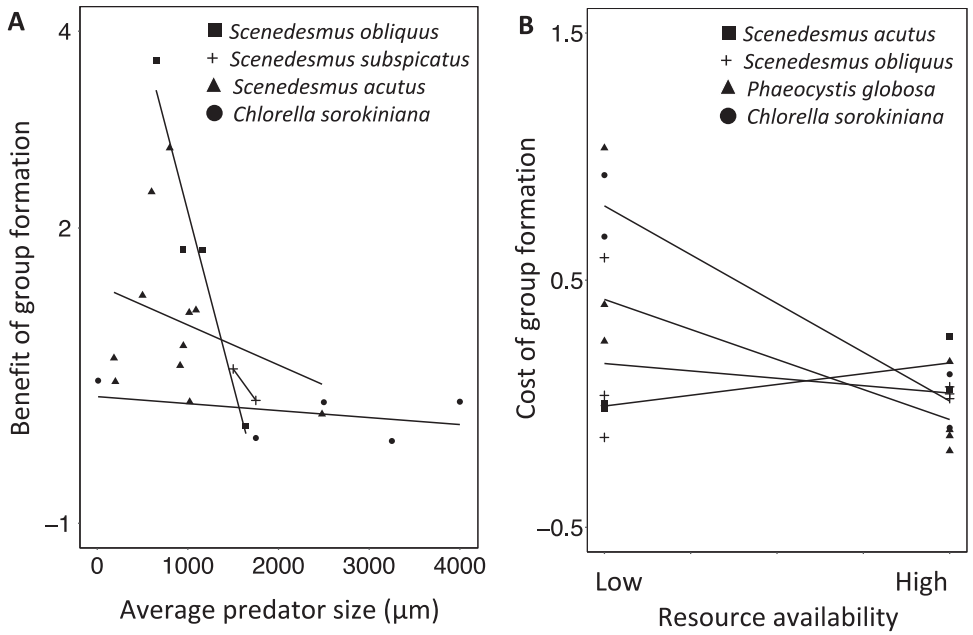

Figure 7. **Benefits and costs of group formation in algae meta-analysis. (A) We found data from four algal species, including this study, where we could examine whether the benefit of being in a multicellular group varies with predator size (see Supporting Information). Each data point represents a different experimental study with a specific value for the benefit of group formation and average predator size for a particular predator–prey combination (total $n = 22$ studies). Values on the *y*-axis represent the log-transformed mean number of multicellular algae/mean number of unicellular algae after adding the predator. Different shapes show the four different algal species, with a regression line shown for each. In all four species, group formation led to a greater reduction in predation against smaller predators. (B) We found data from four algal species, including this study, where we could examine whether the cost of group formation varied with resource availability. Each data point represents a different experimental study with a specific value for the cost of group formation and resource availability for a particular predator–prey combination (total $n = 22$ studies). Values on the *y*-axis represent the log-transformed mean number of unicellular algae/mean number of multicellular algae, thus higher *y* values essentially mean a higher cost of multicellular group formation. Different shapes show the four different algal species, with a regression line shown for each. In three of the four species, group formation was more costly in poorer quality environments.**

reduction of predation by only certain size *D. magna* (Fig. 3B) or by an effect on relative predation rates when different size groups were available (Fig. 3C). Examining the literature we found data from a total of four algal species, including our own study, where the influence of predator size on the benefit of group formation could be examined (Fig. 7A; Fig. S2, Table S1; Hessen & Van Donk 1993; Lürling 1999). In all four cases, the benefit of being in groups was greater against smaller predators (Fig. 7A). Notably, we found a relatively small influence of predator size in *S. subspicatus*, which is the only species out of the four known to produce spines, which may serve as an additional predator defense mechanism apart from group formation (Hessen and Van Donk 1993; Pančić and Kiørboe 2018).

COSTS OF MULTICELLULAR GROUP FORMATION

Previous studies testing for a cost of multicellular group formation have tended to obtain positive results (Lurling and Van Donk 2000; O'donnell et al. 2012; Wang et al. 2014; Wang et al. 2015; Zhu et al. 2015; Zhu et al. 2016). Potential costs of group formation include resource competition, a cost of producing extracellular adhesive molecules, and higher sinking rates (Reynolds 1984; Lancelot and Mathot 1985; Kirk 1994; Trainor 1998; Ploug et al. 1999; Lürling 1999; Tollrian and Dodson 1999). However, lower growth rates of multicellular groups have not been detected in the algae *S. acutus* (Lurling and Van Donk 2000), *Chlamydomonas reinhardtii* (Lurling and Beekman 2006), and *Phaeocystis globosa* (Jakobsen and Tang 2002) in high nutrients. Furthermore, multicellular groups of *Phaeocystis* sp. outcompete single cells in environments high in nitrate and irradiance, rather than grow slower, although formal measurements have not been taken in these studies (Peperzak 1993; Riegman et al. 1992). Multicellular groups have higher sinking rates in *Scenedesmus*, but not in *P. globosa* (Conway and Trainor 1972; Lurling and Van Donk 2000; Jakobsen and Tang 2002).

A possible explanation for the negative results is that the costs only manifest under certain conditions such as when resources are more limiting (Lampert et al. 1994; Boraas et al. 1998; Tollrian and Dodson 1999; Tollrian and Harvell 1999; Lurling and Van

Donk 2000; Jakobsen and Tang 2002). We found that multicellular groups grow at lower rates than single cells, but only when the algae are kept under lower light availability, and hence when competition for light in the tube is greater (Figs. 4C, 5). Examining the literature we found data from four algal species, including our own study, where the influence of resource availability on the cost of group formation could be examined (Fig. 7B; Fig. S2, Table S1; Lürling & Van Donk 1997; O'donnell et al. 2012; Wang et al. 2014; Wang et al. 2015; Zhu et al. 2016). In three out of four species, the cost of being in a group was greater in poorer quality environments (Fig. 7B).

In the absence of predators, we might expect groups to be broken up when competition for resources, and hence the cost of being in a group is greater. However, we did not find that groups broke up more quickly in the dark (Fig. 6). Although we found that the proportion of cells in groups went down in the absence of predators, further work is required to determine if this is due to groups breaking up and/or new cells being predominantly unicellular. Previous studies have suggested that *Scenedesmus acuminatus*, *S. obliquus*, and *C. vulgaris* break up groups quicker in the dark (Dehning and Tilzer 1989; Boraas et al. 1998; Verschoor et al. 2009). This contradiction could reflect different selective regimes, different costs and benefits, mechanistic constraints, and/or differences in the way groups form. For instance, some algae display constant stickiness independent of nutrient availability (Kiørboe et al. 1990; Kiørboe et al. 1994).

MULTICELLULAR GROUP FORMATION AND MAJOR TRANSITIONS

Our results add to a growing body of research showing how multicellular group formation can provide benefits (Grosberg and Strathmann 2007; Michod 2007; Claessen et al. 2014; Lyons and Kolter 2015). In several bacterial species and freshwater algae, such as *C. reinhardtii*, multicellular groups are successful in avoiding predation (Hahn et al. 1999; Matz et al. 2004; Corno and Jürgens 2006; Lurling and Beekman 2006; Queck et al. 2006; Jezberová and Komárková 2007; Yang et al. 2009; Becks et al. 2010; Herron et al. 2018). The mucilaginous matrix of groups can serve as storage for energy and trace elements (Lancelot et al. 1994). Groups of mycobacteria and choanoflagellates are better at foraging (Dworkin and Bonner 1972; Berleman and Kirby 2009; Nichols et al. 2009; Roper et al. 2013), and fruiting bodies of slime molds and *Myxococcus* bacteria are better at dispersal (Velicer and Yuen-tsu 2003; Smith et al. 2014). Groups may be more efficient in using extracellular factors, such as the invertase produced by the yeast *Saccharomyces cerevisiae,* to break down sugars, or the proteases used by bacteria to break down proteins (Koschwanez et al. 2011; Darch et al. 2012; Koschwanez et al. 2013; Biernaskie and West 2015).

Our results also suggest a potential explanation for why group formation may sometimes be facultative. If group formation has a cost, such as increased competition for resources (Fig. 4C), and the benefits only arise at certain times, such as when predators are present (Fig. 2B), then selection could favor group formation to be conditional, in response to cues that signal the benefits. A key future step is to determine why, once groups have formed, cells evolve to perform different tasks (Gavrilets 2010; Ackermann 2015; West and Cooper 2016; Cooper & West 2018). Once this division of labor becomes so extreme, that the different cell types are dependent upon each other, then a major transition can be made to obligate multicellularity, and a new higher level organism (Maynard Smith and Szathmary 1995; Bourke 2011; West et al. 2015).

AUTHOR CONTRIBUTIONS

S.E.K. conducted the study. Both S.E.K. and S.A.W. conceived, designed, analyzed, and wrote the study.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.6g0p74j.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Algae precipitate when not exposed to *Daphnia* supernatant.
Figure S2. PRISMA Flowchat.
Figure S3. Algal growth curves.
Figure S4. Presence of *C. sorokiniana* inside *O. danica*.
Table S1. Excluded studies from the meta-analysis and reason for exclusion.
Movie S1. (https://www.youtube.com/watch?87v=83MAwPoPCCc). This movie shows the flagellate predator *Ochromonas danica* unable to ingest a multicellular group of the algae *Chlorella sorokiniana*.