How do algae form multicellular groups?

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ABSTRACT

Background: Theory suggests that how groups are formed can have a significant influence on the evolution of cooperation, and whether cooperative groups make the major evolutionary transition to a higher-level individual. The formation of clonal groups, by remaining with parents (subsocial group formation), leads to a greater kin selected benefit of cooperation, compared with formation of groups by aggregating, with potential non-relatives (semisocial group formation). Freshwater algae form multicellular groups in response to the presence of predators, but it is not clear whether they form groups by remaining together or by aggregation.


Results: Fluorescence microscopy and time-lapse photography revealed that, in response to predator supernatant/live predators, these algae form groups by both remaining with parents and aggregation. Additionally, different algal species form mixed-species multicellular groups in response to predation.

Conclusion: The observation of aggregation, even between species: (1) emphasizes the likelihood of direct fitness benefits of forming groups to avoid predation; and (2) strengthens the across-species correlation between the method of group formation and whether multicellularity is facultative or obligate.

Keywords: predation, Chlorophyceae, induced defence, aggregation, multicellularity.

INTRODUCTION

There have been at least eight independent major transitions to obligate multicellularity on Earth (Maynard Smith and Szathmary, 1995; Bonner, 1998; Grosberg and Strathmann, 1998, 2007; Bourke, 2011; Fisher et al., 2013). All of these transitions from single cells to an obligate multicellular lifestyle arose from daughter cells remaining attached to their parent cell after division (Raven, 1998; Kirk, 2005; Grosberg and Strathmann, 2007; Michod, 2007; Fisher et al., 2013). This pathway towards social group formation is also known as ‘subsocial’, a term first used to describe the social lifestyle of insects (Michener, 1969; Bourke, 2011). The high degree of relatedness and minimal conflict between members of such a group can favour extreme levels of cooperation, alignment of interests,
and interdependence between members, which are defining features of major transitions in individuality (Hamilton, 1964; Maynard Smith and Szathmary, 1995; Boomsma, 2007, 2009; Fisher et al., 2013; West et al., 2015). In contrast, other species, such as slime moulds and Pseudomonas biofilms, only form multicellular groups facultatively, under certain conditions, and have not made the major transition to obligate multicellularity (West et al., 2015). The formation of these facultative multicellular groups often occurs via cells aggregating together. Because these cells are not necessarily related, group formation via aggregation can lead to more potential for conflict.

Many freshwater algae form multicellular groups in response to predators (Solari et al., 2015; Kapsetaki et al., 2016). However, it is not known if these algae form groups by daughter cells remaining with their parents, or by potentially unrelated cells aggregating together. For example, Boraas et al. (1998) and Lurling and Van Donk (2000) suggested that group formation in Chlorella vulgaris and Scenedesmus obliquus was via daughter cells remaining within the parent cell wall after division, similar to multicellular filament formation in the bacteria Flectobacillus sp. (Corno and Jürgens, 2006), and subsocial palmelloid formation in Chlamydomonas induced by the predator Brachionus (Lurling and Beekman, 2006; Harris, 2009). In contrast, Chlamydomonas forms groups by aggregation in response to the predator Peranema (Sathe and Durand, 2016) and S. obliquus forms predator-induced groups within 1 hour, which is faster than its division time, indicating aggregation (Kapsetaki et al., 2016).

In this study, we determine how three algal species, Chlorella sorokiniana, C. vulgaris, and S. obliquus, form groups in response to the presence of predators. We dyed algae of the same species with two different fluorescent dyes, and then exposed them to either live Daphnia or the supernatant from cultures in which Daphnia had been growing. We have previously shown in all three of these algal species that live Daphnia and/or the supernatant from Daphnia cultures induces group formation (Kapsetaki et al., 2016). The appearance of dichromatic groups, composed of individuals dyed with each colour, would indicate at least some aggregation. We examine group formation caused by both Daphnia and the supernatant from Daphnia cultures, so that we can distinguish between the behaviour of the algae and any aggregation or breaking up of groups that could have been caused by the movement of Daphnia. To further validate our findings, we use an additional technique, time-lapse photography, to observe how single cells form multicellular groups.

MATERIALS AND METHODS

Strains

We maintained the algae Chlorella sorokiniana 211/8K (non-axenic from CCAP), Chlorella vulgaris 211/11B (axenic from CCAP), and Scenedesmus obliquus 276/3A (non-axenic from CCAP) in Bold’s Basal media at 20°C under a light/dark cycle of 16:8 hours using fluorescent illumination. We added 500 µg·mL⁻¹ of the antibiotic rifampicin to 1-mL samples of the C. sorokiniana and S. obliquus cultures, and diluted them 1:300 after 24 hours in Bold’s Basal media (Kapsetaki et al., 2016), to eliminate bacteria in the cultures. We maintained the cultures in 1-litre Erlenmeyer flasks shaking at 220 rpm, a light/dark cycle of 16:8 hours using fluorescent illumination, and a temperature of 20°C before using these cultures in experiments.

As predators, we used Daphnia magna (Sciento, UK), which we fed 5 mL S. obliquus (10⁷ cells·mL⁻¹) every 4–5 days. We maintained the Daphnia in 500-mL jars at 20°C with a light/dark cycle of 16:8 hours.
Fluorescence experiments

Same species

We tested how algae form groups by dyeing two cell cultures of the same species with two different fluorescent dyes, mixing them, and then inducing group formation by adding live predators or predator supernatant. We followed a modified version of the manufacturer's recommended staining procedure (Thermo Fisher Scientific, CellTracker™ Fluorescent Probes). We centrifuged the exponentially growing *C. sorokiniana* at 100 g for 10 minutes and resuspended the pellet in CD-CHO Medium (Gibco, Carlsbad, CA). We then split this culture in equal volumes and added the fluorescent dye CellTracker™ Green BODIPY (final concentration 20 µM) to one culture and CellTracker™ Violet BMQC (final concentration 20 µM) to the other culture. We diluted stock dyes in 10 mM DMSO. We covered the two cultures with aluminium foil and left them shaking at 170 rpm overnight at room temperature, centrifuged both cultures at 100 g for 10 minutes, and resuspended them in Bold's Basal media to remove the dyes.

We sonicated the two algal cultures (10 one-second pulses, amplitude 20%) to break up any groups that may have formed during the dyeing process, diluted both cultures to 10⁶ cells·mL⁻¹, and then mixed them together in a 1:1 volume ratio. We added 4.04 mL of the dyed algae in 50-mL falcon tubes to either 0.96 mL of filtered Bold's Basal media (referred to as media in the remainder of this manuscript), 5 adult *Daphnia*, or 0.96 mL filtered liquid from the *Daphnia* culture (predator supernatant; final concentration of three individuals per millilitre). The filter we used in all experiments had a pore diameter of 0.22 µm. We define ‘predator supernatant’ as anything present in the predator culture that could pass through the 0.22-µm filter. This filtered liquid may contain products released from the predators, and/or products from grazed/ungrazed *S. obliquus*. We replicated each treatment three times. We kept the falcon tube caps loose to allow for oxygenation and randomized the tubes on a rack in an incubator at 20°C with a light/dark cycle of 16:8 hours using fluorescent illumination. After 0 and 24 hours, we tilted the falcon tubes five times to mix the culture, and collected 20-µL samples.

We constructed fluid tunnel slides by placing a cover slip onto two strips of Scotch™ double-sided tape on a microscope slide and pipetting the 20-µL algal samples between the cover slip and the slide. We sealed the coverslip with nail varnish, and imaged the samples using a Zeiss Axio Zoom V16 fluorescence stereoscope (Carl Zeiss, Oberkochen, Germany). As excitation/emission spectra for the violet and green dye, we used 405 nm/475 nm and 488 nm/538 nm, respectively. We took nine images per replicate (9 × 3 = 27 images per treatment), and quantified the proportion of cells in monochromatic groups (number of algal cells in monochromatic groups/total number of algal cells) and dichromatic groups (number of algal cells in dichromatic groups/total number of algal cells). In many cases, the exact number of cells in a three-dimensional group, especially in large groups, was difficult to determine from the two-dimensional images (e.g. Fig. 1), as many cells were ‘hidden in the background’. We counted what we observed in the two-dimensional images.

We followed the same procedure for *C. vulgaris* and *S. obliquus*, but in the case of *S. obliquus* we obtained samples at 48 hours instead of 24 hours, as predator-induced group formation had previously been observed at this time point (Kapsetaki et al., 2016).
Different species

To assess whether different species of algae group together, we followed the same experimental procedure as above, except that in the initial steps we dyed a culture of *C. sorokiniana* with the green dye and a culture of *C. vulgaris* with the violet dye. In the combination *C. sorokiniana* with *C. vulgaris*, we obtained samples at 0 and 24 hours; in *C. sorokiniana* with *S. obliquus* and *C. vulgaris* with *S. obliquus*, we collected samples at 0 and 48 hours.

**Fig. 1.** Representative images of dichromatic groups within (A) and between (B) species. (A) Green- and violet-dyed *Chlorella sorokiniana* form a dichromatic group in the presence of *Daphnia*.
(B) Green-dyed *Chlorella sorokiniana* and violet-dyed *Chlorella vulgaris* form a mixed-species dichromatic group in the presence of *Daphnia*.
**Time-lapse photography**

We also tested how *C. sorokiniana* forms groups using time-lapse photography. We added 4.04 mL of *C. sorokiniana* (initial concentration $10^6$ cells·mL$^{-1}$) to 0.96 mL of filtered *D. magna* water (final concentration of three individuals per millilitre) in a 50-mL falcon tube. We maintained the tube at 20°C with a light/dark cycle of 16:8 hours using fluorescent illumination. After 10 hours, we diluted the culture using Bold's Basal media to a final concentration of $4 \times 10^5$ cells·mL$^{-1}$ and transferred 1 mL of the diluted culture onto a 24-well plate. We placed the 24-well plate at room temperature under a phase-contrast microscope (Nikon ELWD 0.3, 20× magnification, LWD) and set the digital camera (Nikon D300, Japan), which was attached to the microscope, to take photos every minute for a total of 96 hours. We assembled the photos into a movie of 4 frames per second using ‘Time Lapse Assembler’ (v.1.5.3).

From the end of the movie, we randomly chose a cell in a multicellular group and tracked it back in time, stopping at the first instant at which it joined this group. We noted whether it joined the group by aggregation (attaching to a group or pair) or by remaining attached to a mother cell after division. We defined a multicellular group as $\geq 3$ cells in close proximity that could not be distinguished as separate cells. We tracked 50 cells in total, each from a different randomly selected group. Using these 50 cells, we measured the proportion that joined their group by aggregation, the remaining cells joining their group as a result of division from their parent cell. However, we were not able to distinguish whether this was just division as part of their normal life cycle or actual group formation. We also measured the time these cells spent with their parent cell after division.

We followed the same experimental procedure for *C. vulgaris* and *S. obliquus*.

**Statistical analysis**

We performed statistical analyses using R v.3.2.3 (R Development Core Team, 2017). To compare the proportion of cells in monochromatic groups between the media, predator supernatant, and live predators treatments in the fluorescence experiments, we used generalized linear models (glm), specifying the family as quasibinomial to account for overdispersion of the data. We performed the same test to compare the proportion of cells in dichromatic groups between the three treatments.

We tested whether group formation was the result of random aggregation in the fluorescence experiments. Random group formation would lead to the proportion of each colour of cells in groups following a binomial distribution. We used the regression method of Green *et al.* (1982) to compare the observed variance ($V_o$) with that expected from a binomial distribution. The observed variance is given by $s^2(1-r^2)$, where $s^2$ is variance in the number of green cells per group and $r$ is the regression coefficient in the relationship between the number of green cells in a group and group size. The expected variance ($V_e$) is given by $\alpha p(1-p)$, where $\alpha$ is group size and $p$ is the expected proportion of green cells. Specifically, $p$ is $(b+\alpha r)/\alpha$, where $b$ represents the intercept. We tested whether the observed variance was significantly higher than binomial. Under the null hypothesis of random aggregation, the residual statistic, $\chi^2 = (V_o/V_e)/(N-2)$, should come approximately from a chi-squared distribution with $N-2$ degrees of freedom, where $N$ is the number of groups sampled (Green *et al.*, 1982).
Within-species group formation

Consistent with previous results, we found that all three algal species – *C. sorokiniana*, *C. vulgaris*, and *S. obliquus* – formed groups in response to predators/predator supernatant (glm, media vs. predator supernatant and live predators: *C. sorokiniana*, $F = 131.29$, $P < 0.001$, $df = 7$; *C. vulgaris*, $F = 82.07$, $P < 0.001$, $df = 7$; *S. obliquus*, $F = 8.79$, $P = 0.02$, $df = 7$) (Fig. 2). The statistical analysis for each treatment pair is presented in Table S1 (www.evolutionary-ecology.com/data/3099Appendix.pdf).

When we added predators or predator supernatant, the proportion of cells in dichromatic groups, which indicates at least some aggregation, was between 7.1% and 70.8% (Fig. 2). For all three algal species, the proportion of cells in dichromatic groups was higher with predator supernatant or live predators than when just media was added (glm across the three treatments: *C. sorokiniana*, $F = 13.08$, $P = 0.006$, $df = 6$; *C. vulgaris*, $F = 39.79$, $P < 0.001$, $df = 6$; *S. obliquus*, $F = 20.26$, $P = 0.002$, $df = 6$; glm, media vs. predator supernatant and live predators: *C. sorokiniana*, $F = 20.22$, $P = 0.002$, $df = 7$; *C. vulgaris*, $F = 53.14$, $P < 0.001$, $df = 7$; *S. obliquus*, $F = 25.14$, $P = 0.001$, $df = 7$) (Fig. 2).

We also found that the distribution of green cells in groups showed significantly more than binomial variation in all three algal species when exposed to either predator supernatant or live predators, except for *C. sorokiniana* upon exposure to live predators (Table 1). Binomial variation would have been consistent with completely random group aggregation, and so our finding of greater than binomial variation suggests some tendency to form groups with algae of the same colour.

Time-lapse experiments of *C. sorokiniana* in the presence of predator supernatant revealed that of the 50 observed cells, each belonging to a different group, 47 had joined their group by aggregation. In *C. vulgaris* and *S. obliquus*, 31 and 22 of the observed cells respectively had joined their group by aggregation when exposed to predator supernatant. The remaining cells (3 in *C. sorokiniana*, 19 in *C. vulgaris*, and 28 in *S. obliquus*) joined their group as a result of division from their parent cell, although we could not distinguish whether this was simply division as part of their life cycle or actual group formation. These cells spent on average $31.8 \pm 20.6$ hours (mean $\pm$ SEM), $10.8 \pm 2.6$ hours, and $51.6 \pm 4.4$ hours respectively with their parent cell after division.

Between-species group formation

We found that all three combinations of algal species – *C. sorokiniana* with *C. vulgaris*, *C. sorokiniana* with *S. obliquus*, and *C. vulgaris* with *S. obliquus* – formed multicellular groups in response to predators or predator supernatant (glm, media vs. predator supernatant and live predators: *C. sorokiniana* with *C. vulgaris*, $F = 96.12$, $P < 0.001$, $df = 7$; *C. sorokiniana* with *S. obliquus*, $F = 33.02$, $P < 0.001$, $df = 7$; *C. vulgaris* with *S. obliquus*, $F = 57.21$, $P < 0.001$, $df = 7$) (Fig. 3).

After adding predators or predator supernatant, the proportion of cells in dichromatic groups (suggesting some between-species group formation) was between 14.8% and 46.8% (Fig. 3). In all three algal species combinations, the proportion of cells in dichromatic groups was higher with predator supernatant or live predators than when just media was added (glm across the three treatments: *C. sorokiniana* with *C. vulgaris*, $F = 11.10$, $P < 0.001$, $df = 7$; *C. sorokiniana* with *S. obliquus*, $F = 8.79$, $P = 0.02$, $df = 7$; *C. vulgaris* with *S. obliquus*, $F = 131.29$, $P < 0.001$, $df = 7$).
Furthermore, in all three algal species combinations the distribution of green cells in groups showed significantly more than binomial variation when exposed to predator supernatant and live predators: C. sorokiniana with S. obliquus, $F = 27.42$, $P = 0.001$, $df = 7$; C. vulgaris with S. obliquus, $F = 67.91$, $P < 0.001$, $df = 7$ (Fig. 3).

Fig. 2. Within-species group formation. The proportion of cells in groups is plotted in the absence of predators (media), the presence of predator supernatant, and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), and those in groups containing a mixture of violet- and green-dyed cells (dichromatic). The different panels show results for the three different algae species: (A) C. sorokiniana after 24 hours; (B) C. vulgaris after 24 hours; and (C) S. obliquus after 48 hours. The values of the y-axes differ between panels. The error bars are standard errors of the mean for each of these two colour combinations. For all species, we found that the presence of live predators or predator supernatant led to increased group formation and increased proportion of cells in dichromatic groups, suggesting a role of aggregation.
supernatant or live predators (Table 1). This suggests a greater than random propensity to form groups with members of the same species.

**DISCUSSION**

We found group formation in all three algal species – *Chlorella sorokiniana*, *C. vulgaris*, and *Scenedesmus obliquus* – in response to live predators/predator supernatant (Fig. 2), consistent with previous results using these and other algae species (reviewed in Kapsetaki *et al.*, 2016). In all three species, when we dyed algae two different colours and mixed them, we found that they formed dichromatic groups, suggesting that some group formation is via individuals aggregating together (semisocial group formation; Figs. 1A, 2). This result was supported by direct observation in all three species, with time-lapse photography, where we observed individuals coming together. In each of these species, the distribution of dyed cells in groups showed greater than binomial variation, and so group formation was not only due to random aggregation (Table 1). This suggests that either some group formation is via offspring remaining with their parents (subsocial group formation) or that there is some spatial clustering of cells (Table 1). Finally, we found that individuals of these three species also form groups with each other, leading to mixed-species groups, again emphasizing the role of group formation via aggregation (Figs. 1B, 3).

**Table 1.** Comparison of the proportion of green-dyed cells in groups relative to a random binominal variance

<table>
<thead>
<tr>
<th>Algae</th>
<th>Predators</th>
<th>$V_o/V_e$</th>
<th>Number of groups sampled</th>
<th>$\chi^2$-value</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chlorella sorokiniana</em></td>
<td>supernatant</td>
<td>4.81</td>
<td>4</td>
<td>9.62</td>
<td>0.008</td>
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<tr>
<td></td>
<td>live</td>
<td>1.63</td>
<td>3</td>
<td>1.63</td>
<td>0.201</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
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<td>7.49</td>
<td>36</td>
<td>254.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>live</td>
<td>68.20</td>
<td>6</td>
<td>272.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>Scenedesmus obliquus</em></td>
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<td>23.75</td>
<td>221</td>
<td>5201.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>live</td>
<td>55.57</td>
<td>54</td>
<td>2889.77</td>
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</tr>
<tr>
<td><strong>Between species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. sorokiniana</em> and <em>C. vulgaris</em></td>
<td>supernatant</td>
<td>6.22</td>
<td>32</td>
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<td></td>
<td>live</td>
<td>17.90</td>
<td>12</td>
<td>179.03</td>
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<tr>
<td><em>C. sorokiniana</em> and <em>S. obliquus</em></td>
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<td>17.48</td>
<td>24</td>
<td>384.61</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
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<td>28</td>
<td>624.07</td>
<td>&lt;0.001</td>
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<tr>
<td><em>C. vulgaris</em> and <em>S. obliquus</em></td>
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<td>66.78</td>
<td>163</td>
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<tr>
<td></td>
<td>live</td>
<td>39.29</td>
<td>58</td>
<td>2200.58</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Note:* Analyses are shown for when the different coloured cells (green and violet) are the same or different species, and when group formation was induced either by predators or predator supernatant. A value of observed/expected variance ($V_o/V_e$) > 1 would imply overdispersion, where groups tend to show a bias to one of the two colours. In all cases within species and between species, except *C. sorokiniana* in the presence of live predators, mixing was non-random.
Previous studies have suggested that cell division is necessary for group formation (Lampert et al., 1994; Trainor, 1998). *Chlorella sorokiniana*, *C. vulgaris*, and *S. obliquus* acquire energy from sunlight and nutrients in their environment, leading to an increase in cell size, after which the parent cell divides into daughter cells inside the cell wall (Nilshammar and Walles, 1974; Trainor et al., 1976; Boraas et al., 1998; Trainor, 1998; Yamamoto et al., 2005). Then, in response to predation, as reported in *C. vulgaris* and *S. obliquus* (Boraas et al., 1998; Lurling and Van Donk, 2000), daughter cells fail to break free from the parent cell wall, leading to group formation. As stated clearly by

Fig. 3. Between-species group formation. The proportion of cells in groups is shown in the absence of predators (media), the presence of predator supernatant, and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), and those in groups containing a mixture of violet- and green-dyed cells (dichromatic). The different panels show results for the three different algal species combinations: (A) *C. sorokiniana* with *C. vulgaris* after 24 hours; (B) *C. sorokiniana* with *S. obliquus* after 48 hours; and (C) *C. vulgaris* with *S. obliquus* after 48 hours. The values of the y-axes differ between panels. Error bars represent standard errors of the mean for each of these two coloured types. In all three combinations, we found that the presence of live predators or predator supernatant led to increased group formation and increased proportion of cells in dichromatic groups, indicating between-species group formation.
Lürling (2001), in *Scenedesmus* ‘... colony formation is not clogging of individual cells, but the result of a reproductive process’. In contrast to this assumption, we have found that a significant fraction of group formation is via aggregation (Figs. 1, 2). Our results do not exclude the possibility that some group formation occurs through remaining with parents, because group formation is not purely random (Table 1), and with time-lapse photography we observed some cells forming groups by division. However, we could not determine whether this was just division as part of their life cycle or actual group formation (e.g. Appendix – Movie S1). By further analysing our time-lapse data, we found that in all three algal species, daughter cells spent more time on average with their parent cells after division in the presence than in the absence of predator supernatant (Appendix – Time-lapse analysis), although these two treatments were not conducted simultaneously. These observations support the idea of some group formation by remaining with parent cells.

Bonner (1998) suggested that group formation by remaining with parents is more likely to have evolved in aquatic species, whereas we are more likely to see group formation via aggregation in terrestrial species (Bonner, 2003; Velicer and Vos, 2009). Group formation by aggregation has been considered more difficult in water because cells disperse easier in water than on land (Bonner, 2009; Bourke, 2011). How can we explain the group formation by aggregation that we have observed in non-motile aquatic species (Yamamoto et al., 2005)? These algae seem to move at random in the liquid culture, consistent with Brownian motion. In the presence of predator supernatant only, we saw cells dividing and the daughter cells dispersing, cells dividing and the daughter cells remaining with their parent cell, and several cases where a group formed both by cells remaining with parents and by aggregation (Appendix – Movies S2 and S3).

Not only did algae form groups via aggregation, but they also grouped with other species (Fig. 3). This would be expected if rapid group formation provided a direct benefit in defence against predators. Between-species multicellular aggregates have been observed previously in *C. vulgaris* with the bacteria Bacteroidia, Flavobacteria, Beta-proteobacteria, Gamma-proteobacteria, and filamentous blue-green algae (Gutzeit et al., 2005; Lee et al., 2013; Quijano et al., 2017), between different species of *Chlamydomonas* (Sathe and Durand, 2016), and in *Dictyostelium* amoebae (Kausik et al., 2006; Sathe et al., 2010, 2014). Examples of mixed-species multicellular groups also exist in bacterial biofilms, such as *Pseudomonas suringae* with *Pseudomonas agglomerans*, and *Acinetobacter* with *Pseudomonas putida* (Monier and Lindow, 2005; Hansen et al., 2007), where groups may provide protection against grazing by predators (Matz and Kjelleberg, 2005; Chavez-Dozal et al., 2013; Friman et al., 2013).

We found that group formation was not random, either within or between species (Table 1). There are a number of possible explanations for this. First, some group formation could be via remaining with parent cells. For example, the algae *Chlamydomonas* can form groups by both remaining with parents and aggregating (Luling and Beekman, 2006; Harris, 2009; Sathe and Durand, 2016). Second, clumping of the same clone/species might occur just through spatial clustering after division (i.e. limited dispersal in a structured population). Third, individuals might discriminate who they form groups with, as has previously been observed in *Dictyostelium* amoebae (Mehdiabadi et al., 2006).

In conclusion, across species there is a correlation between the method of group formation and whether multicellularity is facultative or obligate (Grosberg and Strathmann, 2007; Fisher et al., 2013, 2016). All the known major transitions to obligate multicellularity have arisen via offspring remaining with their parent cell (Fisher et al., 2013). In contrast, transitions to facultative multicellularity have occurred via both aggregation and remaining with parents (Fisher et al.,
Consequently, our finding that facultative group formation in algae is via aggregation strengthens the across-species correlation between the method of group formation and whether multicellularity is facultative or obligate.

**DATA ACCESSIBILITY**

The data for this paper are available on Dryad (doi: 10.5061/dryad.vb665).

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