Multicellular group formation in algae

Stefania Elisavet Kapsetaki
New College, Department of Zoology
University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
2019

Supervisor: Stuart West
Examiners: Lindsay Turnbull & Andrew Beckerman

Declaration

I declare that this thesis was composed by myself and that the work contained herein is my own except where explicitly stated in the text. This work has not been submitted for any degree or professional qualification except as specified.

Stefania E Kapsetaki

Acknowledgements

I thank everyone who has made the slightest sacrifice for me.

First of all, I thank my parents; without them I wouldn't be here. Thanks to my twin sister for invaluable scientific and other support from day 0, and special thanks to Yovanna Kalpaxi for invaluable ongoing support, especially during the final stages of my PhD. Many thanks to Stu, Ashleigh, and my lab/office mates, Alvaro, Asher, Chucky, David, Gijsbert, Guy, Izzy, Jamie, John, Lorenzo, Mati, Melanie, Miguel, Qi Qin, Sam, Shana, and Tom, for always being extremely supportive in whatever I did, both academically and musically, and in my future job applications.

Thank you Berti for guiding me throughout my Masters project and for being a very present & encouraging scientific voice in my mind during my DPhil, whilst I was in the lab performing experiments with the algae and predators.

Many thanks to Andrew Beckerman, Jags Pandhal, and Nella Roccuzzo for being so friendly, supportive, and open-minded during my visit at the University of Sheffield, despite us working on a very similar, seemingly at first 'competitive', project.

I thank Roni McGowan, Nick Harberd, and Lindsay Turnbull, for providing lab space and assisting my move to the Department of Plant Sciences after the Tinbergen Building closure during mid-February 2017. Thank you Julie Bull and Eric Belfield for making me feel so welcome and comfortable conducting research in my new home!

I'd like to thank Mr. Kotzabasis, Mr. Chalepakis, Mr. Pirintzos, Mr. Oikonomou, Mr. Delidakis, Mr. Apidianakis, Mr. Ladoukakis, Mr. Tzamarias, Mr. Goboitsos, Miss Alexandraki, and Mrs. Koutraki at the University of Crete, for reinforcing the idea of us students making our own world-perspective by critically combining all the knowledge we had gathered from the undergraduate lectures. If it wasn't for the enthusiasm and dedication for science which they conveyed to us students, I probably would not have pursued a path in research.

Last, but not least, I thank my thesis and viva examiners Lindsay Turnbull and Andrew Beckerman, and the A. Onassis and A.G. Leventis Foundations for funding my DPhil studies.

I am not dedicating this thesis to a particular individual. I believe that every scientific work, however big or small, belongs to humanity as a whole.

The End of my doctorate degree … an Ithaka

"As you set out for Ithaka

hope the voyage is a long one,

full of adventure, full of discovery.

Laistrygonians, and Cyclops (mythical giant creatures with a single eye on their forehead – somewhat analogous to *Daphnia* in my study)

angry Poseidon – don't be afraid of them…

Ithaka gave you the marvellous journey.

Without her you would not have set out.

And if you find her poor, Ithaka won't have fooled you.

Wise as you will have become, so full of experience,

you will have understood by then what these Ithakas mean."

Constantinos Cavafis, Greek poet

Publications and Contributions

The following publications have arisen from this thesis and are presented in chapters 2 and 3. The published versions can be found in the appendix.

Chapter 2: Kapsetaki Stefania E., Fisher Roberta M., and West Stuart A. "Predation and the formation of multicellular groups in algae." *Evolutionary Ecology Research* 17.5 (2016): 651-669.

> Stu West, Roberta Fisher, and I conceived, designed, and contributed in writing this study. I collected the data, ran analyses, prepared the figures, and conducted the experiments with the predator products during my DPhil. I conducted the rest of the experiments in this study during my Masters degree.

Chapter 3: Kapsetaki Stefania E., Tep Alexander, and West Stuart A. "How do algae form multicellular groups?" *Evolutionary Ecology Research* 18.6 (2017): 663-675.

> Stu West, Alex Tep, and I conceived and designed this study. Alex Tep also contributed in performing some essential pilot experiments that were not included in this publication. Stu and I interpreted the results and wrote the study. I conducted the experiments, collected the data, ran analyses, and prepared the figures.

The following chapters have not been published.

Chapter 4: Kapsetaki Stefania E. and West Stuart A. “The costs and benefits of multicellular group formation in algae” Submitted at *Evolution.*

> Stu West and I conceived, designed, and wrote this study. I conducted the experiments, collected the data, ran analyses, and prepared the figures.

Chapter 5: Kapsetaki Stefania E. and West Stuart A. "Benefit of algal group formation upon ingestion by the predator *Daphnia*: better ability to kill the predator". *In preparation.*

Stu West and I formulated hypotheses, conceived and designed the experiments, and wrote this study. I conducted the experiments, collected the data, ran analyses, and prepared the figures.

Abstract

The evolution of multicellular organisms from single cells has puzzled scientists for centuries. Separating this problem into smaller steps has been illuminating. The first step is multicellular group formation and the second is group transformation into an obligate multicellular entity where cells are fixed in a multicellular lifestyle and cannot reverse back to being single cells. Relatedness among cells is known to be fundamental in this major transition to an obligate lifestyle, but the role of ecology has not been addressed to such an extent. For instance, in the ecological literature, predation is known to induce defensive group formation in freshwater algae, and this has been mainly investigated as a means of harvesting algae. However, the role of algal predation in the context of multicellularity and social evolution has not been investigated as much. I use the freshwater algae *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus* exposed to the predators *Ochromonas*, *Tetrahymena thermophila,* and *Daphnia magna*, to address *why* and *how* algae form multicellular groups. Answering *why* algae form groups helps us illuminate the costs and benefits involved in this cooperative behaviour. Answering *how* these algae form groups helps us understand whether these algae are likely to make a transition to obligate multicellularity, since we know that all obligate multicellular organisms have evolved via cells remaining stuck to their parent cell after division, not by cell aggregation. We found that these three algal species form multicellular groups in response to the three different predators, and in three predator-prey combinations this multicellular group formation was induced just by predator cues. These multicellular groups formed via aggregation of single cells and by cells remaining attached to the parent cell after division. Also, multicellular groups could form between different algal species. This group formation is costly under conditions of limited resources in

C. sorokiniana, and provides a benefit in terms of avoiding ingestion by small predators, such as *Ochromonos danica,* but not by the larger predator *D. magna*. The benefit of multicellular group formation in the latter case may be that cells in multicellular groups are better able than single cells to kill their predator once they are ingested. Finally, this group formation is reversible. These results indicate that our system has not made a major transition to obligate multicellularity, but has made the first step of facultative multicellular group formation. This facultative feature of our system has therefore been ideal in allowing us to reveal the costs and benefits of multicellular group formation.

Table of Contents

Declaration 2

Acknowledgements 3

Publications and Contributions 5

Abstract 7

Chapter 1: General Introduction 14

Overview 14

Multicellularity in the context of social evolution 14

Abiotic and biotic factors induce group formation 15

Unicellularity, facultative and obligate multicellularity in algae 16

General aim and experimental prey-predator species 17

Chapter aims and objectives 22

Chapter 2: Predation and the formation of multicellular groups in algae* 24

Abstract 25

Introduction 26

Materials and methods 28

Species and growth conditions 28

Experiment 1: Testing for group formation and size change upon exposure to predators . 29

Experiment 2: Testing for group formation upon exposure to predator products 30

Experiment 3: Testing for predation by measuring algal density 34

Experiment 4: Testing for predation by observing ingestion 35

Statistical analysis 36

Results 37

Do algae form groups in response to predators? 37

Does group size change? 38

Do algae form groups in response to predator products? ... 40

Do the predators impact algal density? ... 40

Predator behavioural observations ... 41

Discussion ... 47

Response to predators ... 47

Is group formation a behavioural response of the algae? ... 48

Predation ... 50

Acknowledgements ... 51

Data Accessibility ... 51

Chapter 3: How do algae form multicellular groups?* ... 53

Abstract ... 54

Introduction ... 55

Materials and Methods ... 56

Strains ... 56

Fluorescence experiments ... 57

Time-lapse photography ... 59

Statistical analysis ... 60

Results ... 61

Within-species group formation ... 61

Between-species group formation ... 65

Discussion ... 68

Data accessibility ... 71

Acknowledgements ... 71

Appendix ... 71

Chapter 4: The costs and benefits of multicellular group formation in algae* ... 74

Abstract 75

Introduction 76

Methods 79

Strains *79*

Experiment 1: Is there a fitness benefit of being in a group? *80*

Experiment 2: Is there a fitness cost of being in a group? *85*

Experiment 3: Do groups revert to unicellularity faster in the dark? *86*

Meta-analyses *86*

Statistical Analysis *88*

Results 89

Experiment 1: Is there a fitness benefit of being in a group? *89*

Experiment 2: Is there a fitness cost of being in a group? *94*

Experiment 3: Do groups break up faster in the dark? *97*

Meta-analyses *98*

Discussion 100

Multicellular group formation in algae *101*

Multicellular group formation and major transitions *104*

Authors' contributions 105

Acknowledgements 105

Competing interests 105

Supplementary Material 106

Chapter 5: Benefit of algal group formation upon ingestion by the predator *Daphnia*: better ability to kill the predator 109

Abstract 110

Introduction 110

Methods 115

Strains *115*

Observations of algae on the sides of tubes and Scanning Electron Microscopy imaging *116*

Testing whether algae can survive inside the Daphnia's gut *118*

Testing whether algae can survive passed the Daphnia's gut *119*

Statistical analysis *120*

Results 121

Predator products induce algal group formation and stickiness of algal groups to plastic *121*

Daphnia can remove and ingest the sticky green material from the sides of tubes *122*

Algae can be alive inside the Daphnia's gut *122*

Algal survival through Daphnia's gut and higher Daphnia death rates when fed multicellular algae *122*

Discussion 126

Survival inside and passed the predator's gut *126*

Survival passed the predator's gut as a means of prey dispersal *127*

Multicellular groups are better able than single cells to kill their predator *128*

Acknowledgements 130

Supplementary Material 130

Chapter 6: General Discussion 131

Overview 131

Summary of main results 131

Clarification: What is a predator? 131

Group formation: additional proximate explanations 132

Predatory group-inducing cues *132*

Predator-induced group-adhesive substance *133*

Group formation: additional ultimate explanations 136

Clump: why reverse? *136*

Why be plastic versus fixed? *136*

What abiotic factors favour multicellularity? 138

Concluding remarks 139

References 140

Appendix 165

Chapter 1: General Introduction

Overview

In this chapter I discuss multicellularity in the context of social evolution, and explain ecological factors known to induce multicellular group formation. Then I provide a summary of the various unicellular, facultatively and obligately multicellular algae. Next, I discuss the general aim of the thesis and the predator-prey species of our choice. Finally I describe the specific aims of each data chapter.

Multicellularity in the context of social evolution

The first cell on Earth arose approximately 3500 million years ago, and the first eukaryotic cell approximately 1500 million years later. It then took another 800 million years for the first multicellular eukaryotes to appear (Bourke 2011). This change from one level of individual, the single cell, to a new higher-level individual, the obligate multicellular organism, is termed a major evolutionary transition in individuality (Maynard Smith and Szathmary 1995; West et al. 2015). To better identify and understand the evolutionary forces driving this transition in individuality, Andrew Bourke (2011) highlighted the usefulness of viewing such major transitions as distinct steps. The first step is social group formation, where single cells form a cooperative group, which can be reversible, and is sometimes called a minor evolutionary transition. This step can be driven by several ecological factors which I discuss in the following paragraph. The second step is social group transformation, where the fitness interests of cells are aligned, cells cannot reproduce independently anymore, and division of labour usually appears (Maynard Smith and Szathmary 1995; Queller 2000; Bourke 2011; West et al. 2015). This second step is most often achieved by offspring-attachment to the parent cell leading to high levels of relatedness, and thus limiting genetic conflict among cells (Grosberg and Strathmann 1998; Grosberg and Strathmann 2007). All known obligate multicellular organisms have evolved from this mechanism of cells remaining attached to their parent cell after division, not by cell aggregation (Raven 1998; Fisher et al. 2013; Tarnita et al. 2013). Social group transformation is irreversible. Once the multicellular group has reached this step, it has become an obligately multicellular organism; it can no longer reverse back to a single-cell independent lifestyle, and fits the definition of a major evolutionary transition. Importantly, I must clarify that this major transition perspective does not claim that the switch from single cells to a multicellular organism is inevitable. In other words, there is no directional bias in evolution for an increase in complexity (Bourke 2011). Even when single cells have made the first step of cooperative group formation, this does not necessarily mean that they are *en route* towards a major transition.

Abiotic and biotic factors induce group formation

The first step of multicellular group formation can be induced under certain ecological conditions, abiotic and biotic. Group formation can be induced by several chemicals, such as glucose, calcium, strontium, glycerophosphate, ammonium, glycolic acid, components in diesel-fuel oil, anionic surfactants (which neutralise the surface charge of algal cells causing them to aggregate), urea, low phosphate, low salt concentrations, and low temperatures (Kylin and Das 1967; Shubert and Trainor 1974; Monahan 1977; Siver and Trainor 1981; Egan and Trainor 1989; Trainor 1992; Trainor 1993; Tukaj and Bohdanowicz 1995; Wiltshire et al. 1999; Lürling 1999). Regarding biotic factors, group formation can be induced when prey are exposed for example to predators. Group formation is part of an arsenal of pre- and post-ingestion anti-predator defences in bacteria and algae, together with other chemical (luminescence, excretion of toxins that paralyse/kill the predators), life history (vertical migration, different timing of algal recruitment) and morphological defences (spine production, increase in cell size, thicker cell wall, exopolymer transparent substance possibly hindering predator digestion) (Porter 1973; Porter 1975; DeMott et al. 1991; Hessen and Van Donk 1993; Christoffersen 1996; Lass and Spaak 2003; Verschoor et al. 2007; Van Donk et al. 2011; Pančić and Kiørboe 2018). The assumed benefit of group formation upon exposure to predators is that groups are too large for predators to ingest, or if ingested, groups survive digestion through the predator's gut (Ryther 1954; Porter 1976; Hessen and Van Donk 1993; Lürling 1999).

Unicellularity, facultative and obligate multicellularity in algae

Obligate multicellularity has evolved at least eight times independently throughout the tree of life; once in ciliates, animals, plants, fungi, and four times in algae (Knoll 2011; Fisher et al. 2013; Niklas and Newman 2013; Lyons and Kolter 2015). Specifically in algae, obligate multicellularity has risen four times independently in the brown algae, red algae, cyanobacteria, and Volvocine algae (J. Bonner 2009; Fisher et al. 2013). However, this does not mean that all members of these taxa are obligately multicellular. Algae include unicellular, facultatively and obligately multicellular species. For instance, the Chlorophyte green algae consist of unicellular (e.g. *Chlorella singularis, Parachlorella kessleri*), facultatively multicellular (e.g. *Chlorella vulgaris, Scenedesmus obliquus, Chlamydomonas reinhardtii* from the Volvocine algae), and obligately multicellular species (e.g. *Dictyosphaerium pulchellum*, *Mucidosphaerium pulchellum, Volvox carteri*) (Hoek et al. 1995; Lürling 1998; Umen 2014; Krienitz et al. 2015; Fisher et al. 2016). In fact, algae are considered as one of the most diverse taxa in nature (Whitton et al. 2002). Their tissues can be simple/undifferentiated/differentiated and extremely morphologically complex. Their size can range from cells of a few micrometers to as long as one hundred meters, across freshwater, terrestrial, and marine environments (Round 1965). They also vary in their intracellular and extracellular biochemical composition, with diverse cell walls, lipid/carbohydrate contents, and pigments, and even in their motility, as some species are non-motile, others amoeba-like, and others motile (Round 1965; Whitton et al. 2002). According to Baker (1948), the fact that such photosynthetic organisms depend on the sun for acquisition of food, and thus there was probably no selection for a specific feeding mechanism, may explain the huge diversity in shapes, sizes, and cell contents that we observe in algae. Still, our knowledge of what caused the morphological leap to complexity is limited. We need a more solid understanding of what causes seemingly simple single cells to become a more complex form, a multicellular group.

General aim and experimental prey-predator species

The general aim of this thesis is to answer *why* and *how* single-celled algae form multicellular groups. These two questions are famously known as the ultimate (fitness consequences) and proximate (mechanistic) explanations of a behaviour, respectively (Mayr 1961; Tinbergen 1963; Kruuk 2003; Bateson and Laland 2013). These perspectives are not competing. On the contrary, together they provide a fuller understanding of an organism's behaviour, e.g. group formation. As our model system we chose the freshwater algae *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus*, upon exposure to the predators *Ochromonas*, *Tetrahymena*, and *Daphnia*.

The freshwater Chlorophyte algae have been in existence and dividing for over two billion years (Knoll 2003; Teyssèdre 2006). *Chlorella* and *Scenedesmus* are among the most common genera in this taxon (Trainor 1998). They are immotile, eukaryotic, and mainly unicellular. Cells generally divide via two nuclear divisions inside the parent cell, and exit through an opening in the cell wall, giving rise to four offspring. The outcoming cells may either separate becoming unicells or stay together as a colony (Hoek et al. 1995; Yamamoto et al. 2004; Yamamoto et al. 2005). We chose *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus* as our model system since they belong to a taxon, the Chlorophytes, where a major transition to multicellularity has occurred, and therefore they are potentially a good model for elucidating the first step in the evolution of multicellularity; group formation. Another reason we chose these unicellular/facultatively multicellular species is because of their flexibility in their ability to form groups enabling us to measure under which circumstances they may form groups and measure the costs and benefits of group living.

Ochromonas is a motile, freshwater, single-celled, spherical, cylindrical to pyriform in shape, phototactic, mixotrophic and heterotrophic predator. It has two unequal flagella. The long flagellum creates currents in the liquid that bring food particles to the cell membrane, to the base of the flagellum, where particles are engulfed via phagocytosis (Fenchel and Kinne 1987; Sleigh 1989). *Ochromonas* is known to be a size-selective feeder, readily ingesting prey of 4-15 μm in diameter, but rejecting smaller ones (Lu 1988; Seale et al. 1990; Boraas et al. 1998).

Tetrahymena is a motile, freshwater, unicellular, ovoid to pear shaped, phototactic ciliate, uniformly covered by around 500 cilia (Kim et al. 2009). Its doubling time is around 2-3 hours, and it acquires food into its gullet via phagocytosis (Collins and Gorovsky 2005; Orias et al. 2011).

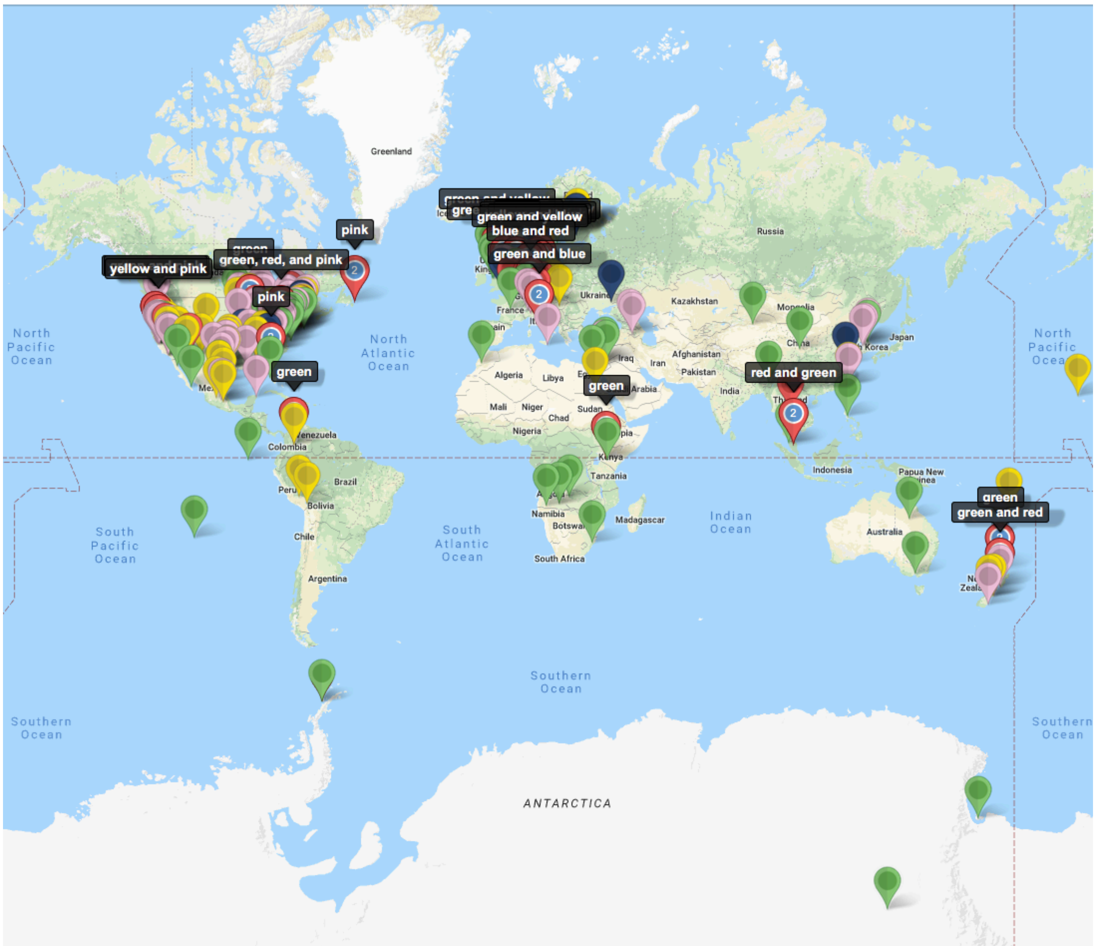
Daphnia is a motile, freshwater, phototactic, multicellular crustacean of a few millimeters in length (De Meester and Dumont 1988; De Meester and Dumont 1989).

Eggs hatch into juveniles when their mother molts, and the juveniles then go through a series of molts and instars before reaching adulthood (Clare 2002; Ebert 2005). *Daphnia*'s life span is approximately eight weeks, although it varies with environmental factors such as temperature, oxygen levels, and food availability, with unstable conditions usually leading to shorter lifespans (Pietrzak, Bednarska, et al. 2010; Pietrzak, Grzesiuk, et al. 2010). Visual senses, like their compound eye allow them to identify light and movement, while their olfactory senses allow them to locate food, relatives, and potential predators (Young 1974; Consi et al. 1990; Roozen and Lürling 2001). In their natural environment, during the day they move to deeper layers of water to avoid predators, while at night they move to upper layers grazing on phytoplankton, bacteria, detritus, and fungal spores (Hanski and Ranta 1983; Gliwicz 1986; Ebert 2005; Coors et al. 2009). Their body is surrounded by a transparent shell-like structure, the carapace, thus making visible what they have eaten (Clare 2002; Ebert 2005). Upon eating green algae, *Daphnia* become transparent with a tint of green/yellow (Hartmann 1985; Meise et al. 1985; Knisely and Geller 1986). *Daphnia* have a multitude of filter feeding structures, hidden in the carapace, constantly moving, and thus difficult to directly observe (Hartmann and Kunkel 1991). Filtration rates may vary with temperature, body size, food density/quality, oxygen concentration, and pH. Appendages in the carapace produce water currents, and setae on their legs filter the food particles, before reaching their mouth, esophagus, midgut, and hindgut (Hanski and Ranta 1983; Roozen and Lürling 2001; Ebert 2005). *Daphnia* display selectivity on certain food depending on food concentrations, and the age and size of *Daphnia*. For instance, *Daphnia* prefer smaller and flagellate algae rather than large spherical and coccoid algae (Hartmann 1985; Meise et al. 1985; Bern 1990).

We chose to examine algal group formation upon exposure to a biotic factor, the above-mentioned predators *Ochromonas*, *Tetrahymena, Daphnia*, since the two former predators are of similar size to the algae, and thus a size advantage of group formation may exist, though in the larger predator *Daphnia* such size benefit may not exist. The use of predators belonging to a range of different taxa also makes our predator-prey system more taxonomically general. Furthermore, these algae have been found to co-exist worldwide with such predatory beasts, and thus this biotic pressure may be an ecologically plausible scenario.

These five prey and predator genera are broadly distributed around the world (Figure 1.1; Trainor 1998). In several locations the prey and predators co-exist indicating that our system has ecological relevance (Figure 1.1). Still, one must be cautious with this interpretation because even if two genera have been found in the same location, they may have been observed/isolated at different points in time. For instance, *T. thermophila* is usually abundant during a specific period, June till September (Doerder et al. 1996). Therefore a shared location in this map does not directly imply co-existence.

(A)


Greenland
pink
yellow and pink
green, red, and pink
green and yellow
blue and red
green and blue
green
red and green
green and red
North Pacific Ocean
North Atlantic Ocean
South Pacific Ocean
South Atlantic Ocean
Indian Ocean
Southern Ocean
ANTARCTICA
Russia
Kazakhstan
Mongolia
China
Japan
Korea
Afghanistan
Pakistan
India
Iran
Iraq
Ukraine
France
Algeria
Libya
Mali
Niger
Chad
Nigeria
Sudan
Kenya
Tanzania
Madagascar
Namibia
South Africa
Colombia
Brazil
Bolivia
Chile
Argentina
Indonesia
Australia

(B)


pink
green
green, red, and pink
green and pink
yellow and pink
United States
Mexico
WASHINGTON
OREGON
IDAHO
MONTANA
WYOMING
NORTH DAKOTA
SOUTH DAKOTA
NEBRASKA
NEVADA
UTAH
COLORADO
KANSAS
MISSOURI
ILLINOIS
INDIANA
MICHIGAN
OKLAHOMA
ARKANSAS
TENNESSEE
MISSISSIPPI
ALABAMA
LOUISIANA
NEW MEXICO
Kansas City
Indianapolis
Nashville
Atlanta
Charlotte
Toronto
Montreal
Quebec City
Detroit
Las Vegas
Los Angeles
San Diego
San Jose
Phoenix
Tucson
El Paso
Houston
San Antonio
New Orleans
Orlando
Miami
Monterrey
Guadalajara
Havana
Cuba
Bermuda
The Bahamas
Haiti
Dominican Republic
Puerto Rico
San Juan
NOVA SCOTIA
NEW BRUNSWICK

(C)

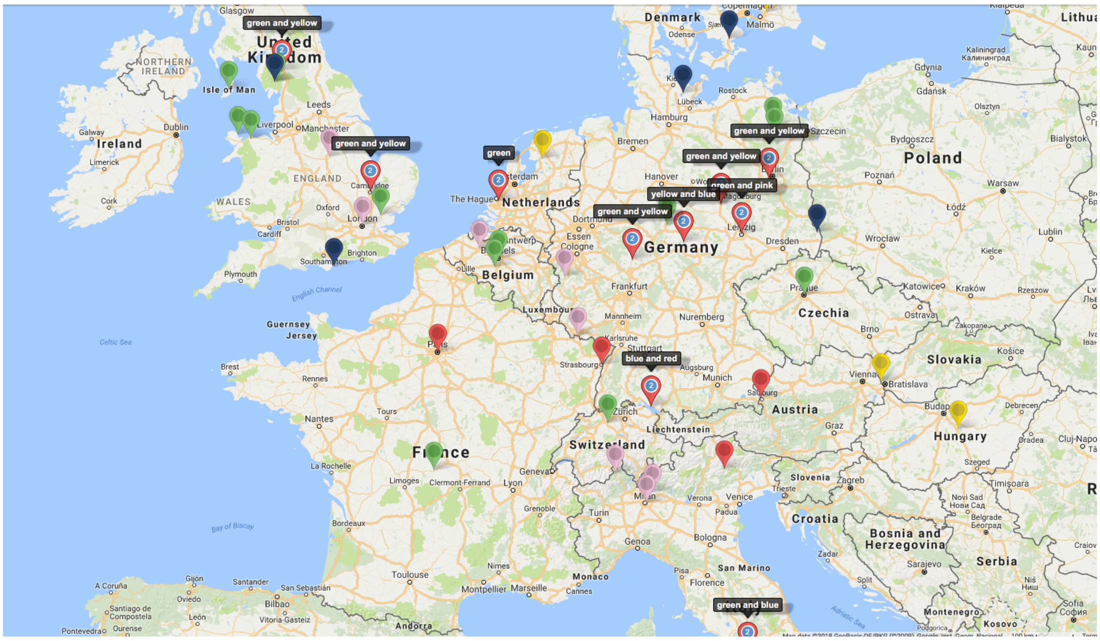


Figure 1.1. Worldwide distribution of *Chlorella* (green), *Scenedesmus* (yellow), *Ochromonas* (blue), *Tetrahymena* (red) and *Daphnia* (pink) (A). There are several locations where the predators and prey appear in the same location. In locations where two or more genera were found, we describe the colours above the marker. Zoomed-in sections of the USA (B) and Europe (C) provide more detail. We made this map using habitat information from UTEX (https://utex.org), iNaturalist (https://www.inaturalist.org), CCAP (https://www.ccap.ac.uk), and the mapping tool fortress.maptive.

Chapter aims and objectives

In chapter 2 I examine whether the algae *C. sorokiniana*, *C. vulgaris*, and *S. obliquus* form multicellular groups in response to live *Ochromonas spp.*, *T. thermophila*, and *D. magna*, and/or their supernatant. If algae form groups in response to predator supernatant, this would indicate that group formation is an algal behavioural response, and not just selective grazing on single cells, or movement of the predators causing grouping. I also test whether *Ochromonas*, *T. thermophila,* and *D. magna* cause a significant reduction in algal densities, an indication of predation.

My findings that algae form groups in response to predators led me to further explore how algae form groups in response to predation in chapter 3. Groups may form by algae remaining together after division and/or by aggregating. The way in which algae form groups is important in the evolution of cooperative behaviours and whether groups make the major transition to multicellularity, since group formation by remaining with parents leads to a higher kin selected benefit than aggregating with potential non-relatives.

In chapter 4 I experimentally quantify the benefit of group formation when the algae *C. sorokiniana* are exposed to the small predator *Ochromonas* and the larger predator *D. magna,* and also the cost of group formation. Furthermore, I conduct a meta-analysis to test whether the benefit of group formation in algae is higher in the presence of smaller predators, and whether the cost of group formation is higher under limited resources.

My observation that *C. sorokiniana* forms groups in response to the larger predator *D. magna* even though there appears to be no survival benefit, since groups are ingested as well as single cells by the *Daphnia*, led me to ask why algae form groups in the presence of *Daphnia.* In chapter 5 I examine whether group formation in *C. sorokiniana* leads to higher algal group survival rates passed the gut of *D. magna* and/or improved ability to kill the predator once ingested.

Chapter 2: Predation and the formation of multicellular groups in algae*

When making these drawings (2nd drawing in chapter 3) almost ten years ago by simply drawing one by one small circles next to each other, little did I know that this would be the focus of my DPhil research… multicellular group formation.

*Published as Kapsetaki S. E., Fisher R. M., and West S. A. "Predation and the formation of multicellular groups in algae." *Evolutionary Ecology Research* 17.5 (2016): 651-669 (appended).

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).

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; Koschwanez et al. 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. 2.1). 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^{o}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 ~10^6 cells · mL^{-1}.

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^{o}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.

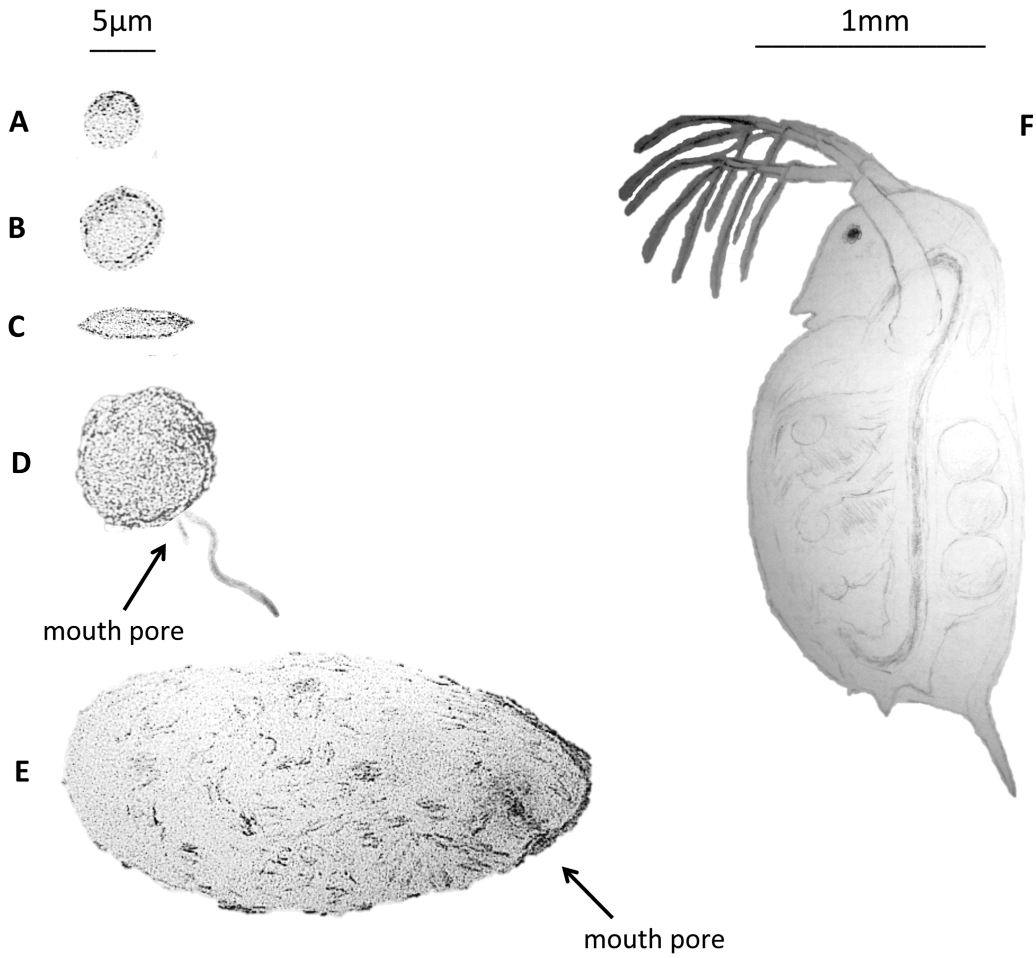


Fig. 2.1. The species used in the present study: (A) *Chlorella sorokiniana*, (B) *Chlorella vulgaris*, (C) *Scenedesmus obliquus*, (D) the flagellate *Ochromonas*, (E) the ciliate *Tetrahymena thermophila*, and (F) the crustacean *Daphnia magna*.

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^{o}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 2.1.

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^{o}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 2.1. 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 2.1. 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	1	2×10^6	1×10^5	20	30*
Ochromonas spp.	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	1	1×10^5	6×10^5	20	9
Ochromonas spp.	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	1, 3	2×10^5	3×10^5	5	9
Ochromonas spp.	2	1×10^6	2×10^4	5	9
	4	1×10^6	2×10^4	1	42
C. sorokiniana with	1	3×10^6	1×10^4	20	30*
T. thermophila	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	1	2×10^6	2×10^4	20	30**
T. thermophila	2	1×10^6	3×10^6	5	9
	3	2×10^5	4×10^5	5	9
	4	4×10^6	9×10^4	1	42
S. obliquus with	1	2×10^5	4×10^5	5	9
T. thermophila	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	1, 3	9×10^6	1	5	9
D. magna	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	1, 3	5×10^6	1	5	9
D. magna	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	1, 3	2×10^6	1	5	9
D. magna	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^{o}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^{o}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 form the algal concentrations used, which we describe in Table 2.1.

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^{o}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 · 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 2.1.

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^{o}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 2.1.

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^{o}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 2.1.

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. 2.2; 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. 2.2A–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% of cells before the *Daphnia* were added, to 80 ± 3% of cells just one hour after the *Daphnia* were added (Fig. 2.2I; glm at 'time point 1 hour', $F = 202.7$, $P < 0.0001$).

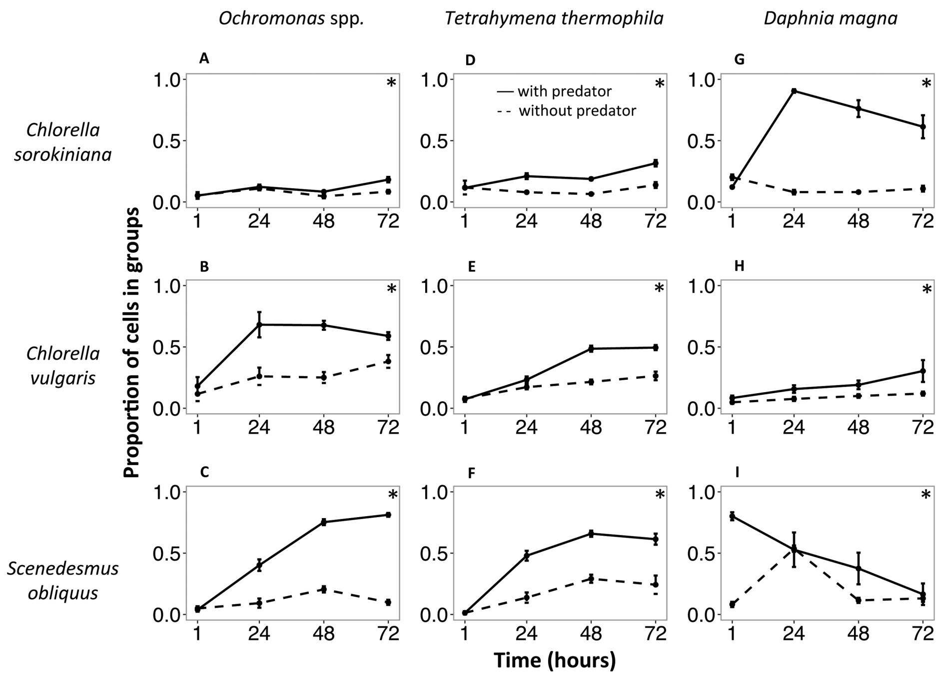


Fig. 2.2. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In all nine combinations, the proportion of cells in groups was higher in the presence of the putative predator [A: generalized linear model (glm), $F = 11.93$, $P < 0.01$; 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.

Does group size change?

Group size increased in the presence of the predator in seven combinations (Figs. 2.3C–I and 2.4C–I). For example, in the *S. obliquus* with *D. magna* combination, the mean group size increased from 3.3 ± 0.1 before the *Daphnia* was added, to 9.7 ± 0.8 just one hour after adding the *Daphnia* (Fig. 2.3I; 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. 2.5). In *Chlorella*, groups were irregularly shaped. In *Scenedesmus*, groups varied in size and morphology – for example, we observed four-celled (Fig. 2.5E) and eight-celled groups, where cells were attached sideways, as well as chain-like groups, where cells were attached by their ends (Fig. 2.5C, D).

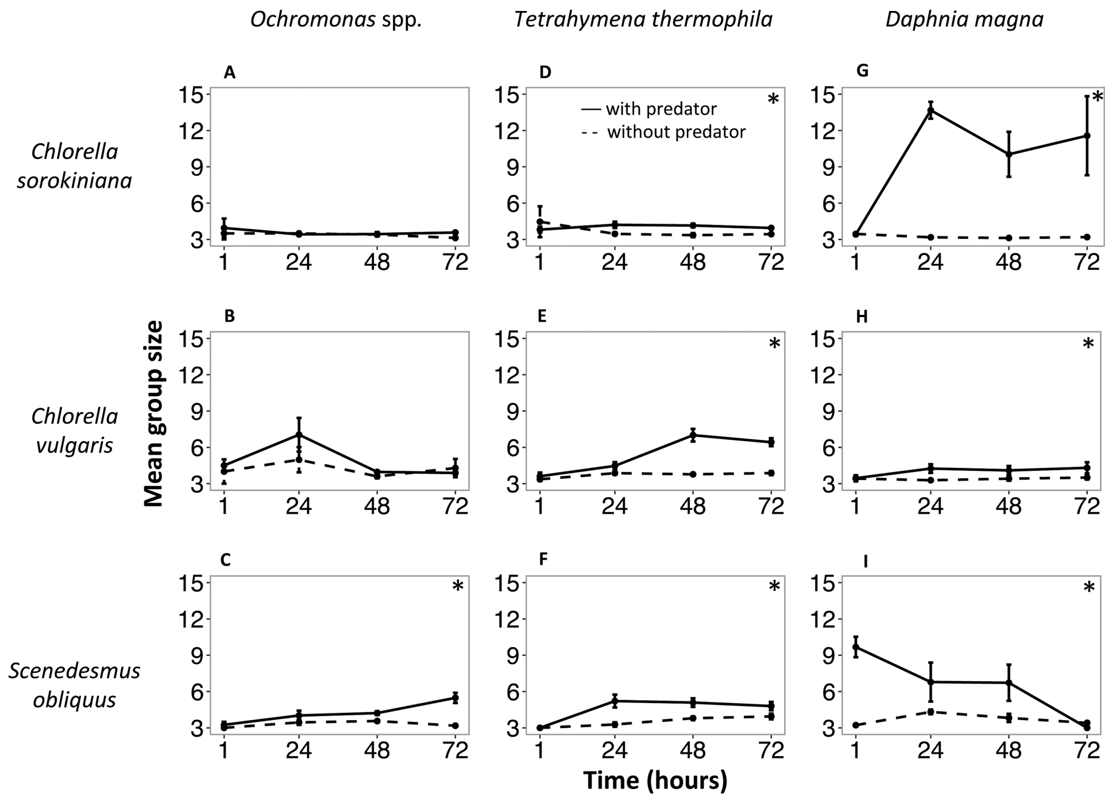


Fig. 2.3. Mean group size plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In seven combinations, the mean algal group size was higher in the presence of the putative predator (C: glmmPQL, $P < 0.0001$; D: glm, $F = 11.48$, $P < 0.01$; E: glm, $F = 63.39$, $P < 0.0001$; 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 predators (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.

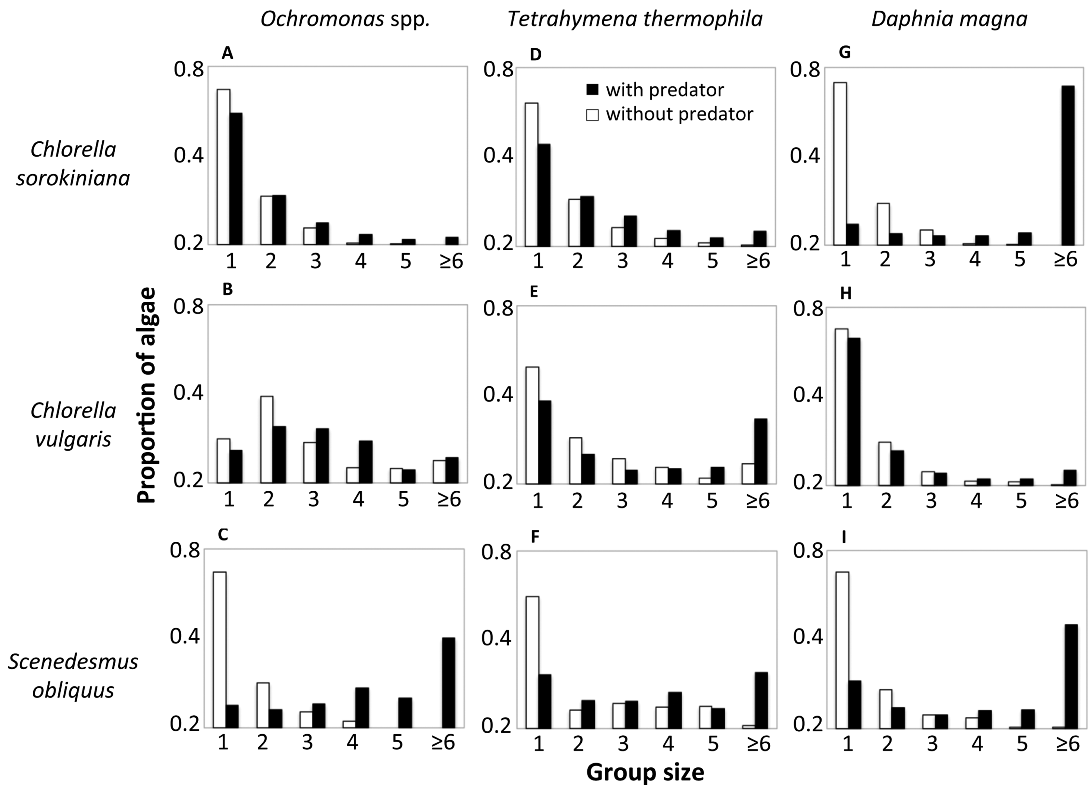


Fig. 2.4. Distribution of group sizes in the presence (solid bars) and absence (open bars) of the putative predator after 72 hours (A–F) and 48 hours (G–I). Group sizes '1' and '2' refer to a unicell and a paired cell, respectively.

Do algae form groups in response to predator products?

In three combinations – *C. vulgaris* with *T. thermophila* (Fig. 2.6a.E), *C. sorokiniana* with *D. magna* (Fig. 2.6a.G), and *S. obliquus* with *D. magna* (Fig. 2.6b.I) – algae formed groups in response to predator products. In the experiments conducted in tube cultures, we observed group formation in two combinations (Figs. 2.6a.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. 2.6b.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. 2.7A, B, G–I). The four combinations in which we did not observe a decrease in algal density were: *S. obliquus* with *Ochromonas* spp. (Fig. 2.7C), *C. sorokiniana* with *T. thermophila* (Fig. 2.7D), *C. vulgaris* with *T. thermophila* (Fig. 2.7E), and *S. obliquus* with *T. thermophila* (Fig. 2.7F).

Predator behavioural observations

In relation to *Ochromonas*, 9.5% of the time (4/42 observations) we observed *Ochromonas* capturing *C. sorokiniana* (Fig. 2.8A); 7.1% of the time (3/42) capturing *C. vulgaris* (Fig. 2.8B); and none of the time (0/42) *Ochromonas* exhibiting any ingesting activity towards *S. obliquus*. Regarding *Tetrahymena*, 7.1% of the time (3/42) *Tetrahymena* ingested *C. sorokiniana* (Fig. 2.8D); 80.9% of the time (34/42) *C. vulgaris* algae were visible inside *Tetrahymena* (Fig. 2.8E); and 2.3% of the time (1/42) *S. obliquus* was seen inside *Tetrahymena* (Fig. 2.8F). Considering *Daphnia*, 100% of the time (3/3) *Daphnia*'s gut was green in the presence of the algae (Figs. 2.8G–I).

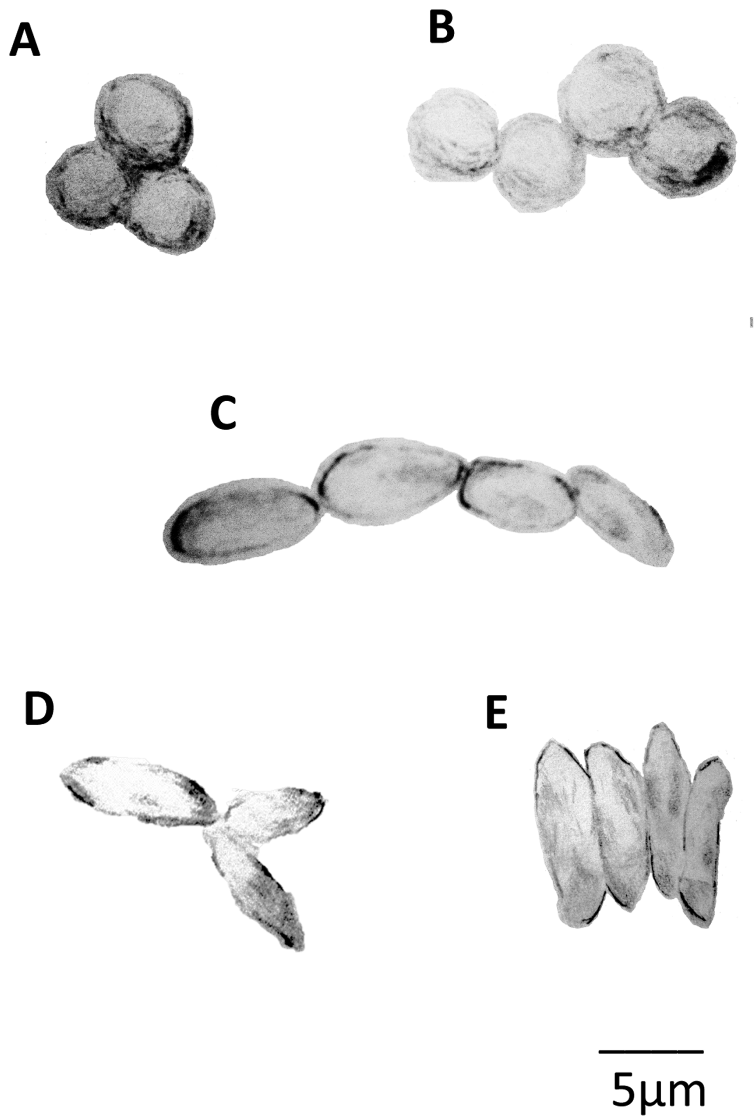


Fig. 2.5. Characteristic algal group types: (A) three-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (B) four-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (D) three-celled group observed in *S. obliquus* cultures; (C, E) four-celled groups seen in *S. obliquus* cultures.

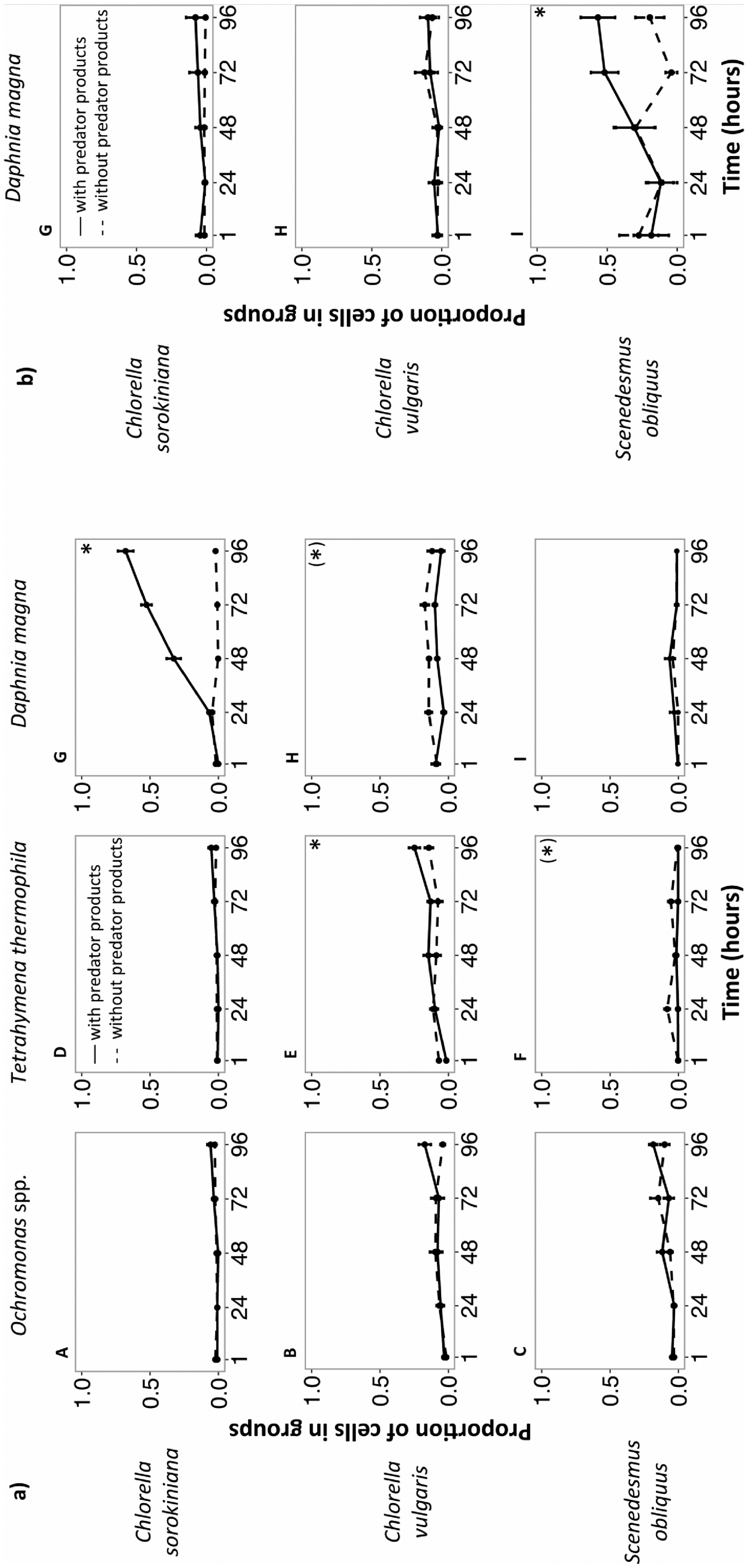


Fig. 2.6. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of predator products. (a) Experiment in tube cultures. In

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 predators (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.

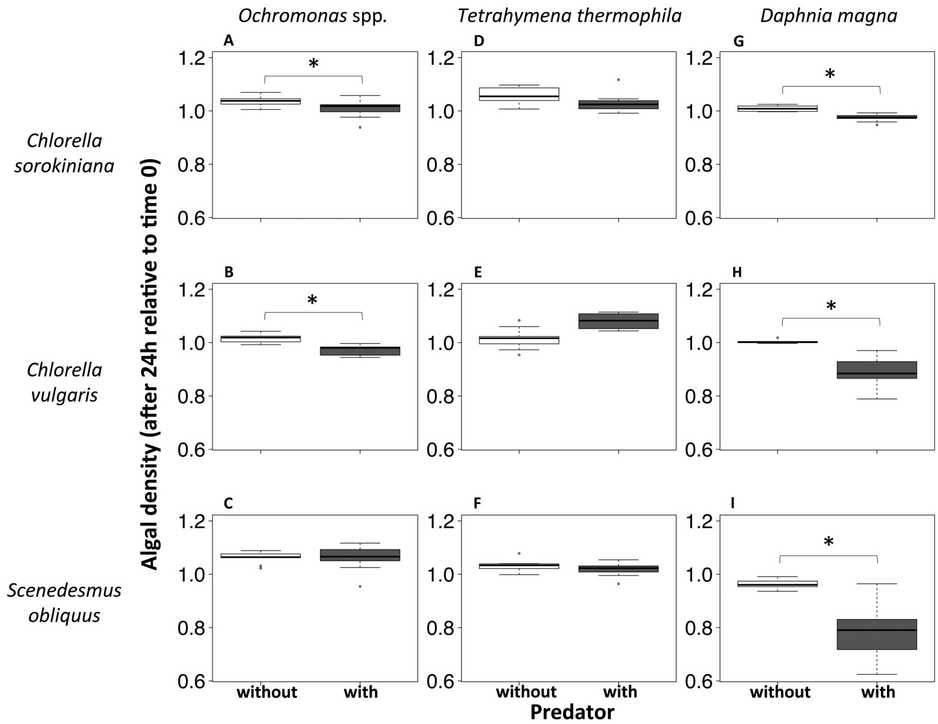


Fig. 2.7. Algal density (after 24 hours relative to time 0) in the presence (grey) and absence (white) of the putative predator. In five combinations, the algal density decreased in the presence of the putative predator (two-sample *t*-test, A: d.f. = 16, *P*

= 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 predators (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.

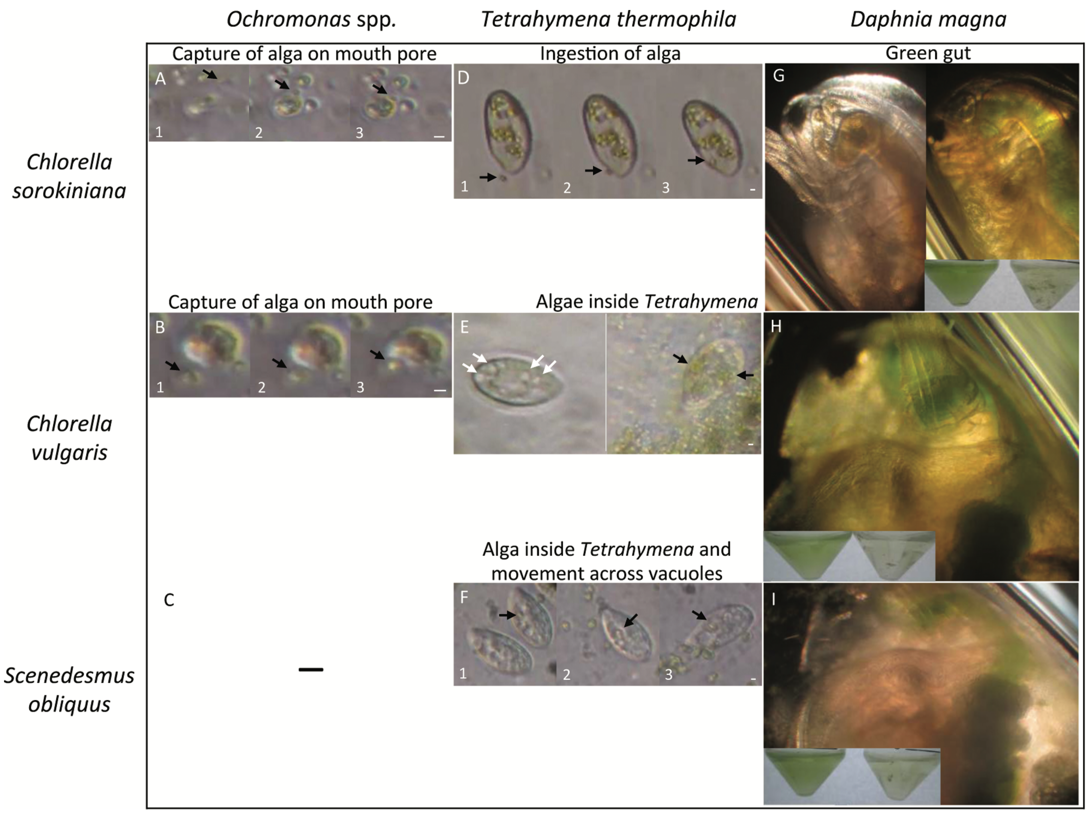


Fig. 2.8. Direct observations of protists' feeding behaviour and *Daphnia*'s gut. Images A, B, and D–F are video snapshots. Black arrows show algae. (A) Capture of unicellular *C. sorokiniana* by *Ochromonas*. *Chlorella sorokiniana* rotates upon contact with *Ochromonas*'s mouth pore and flagella (A2); 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*).

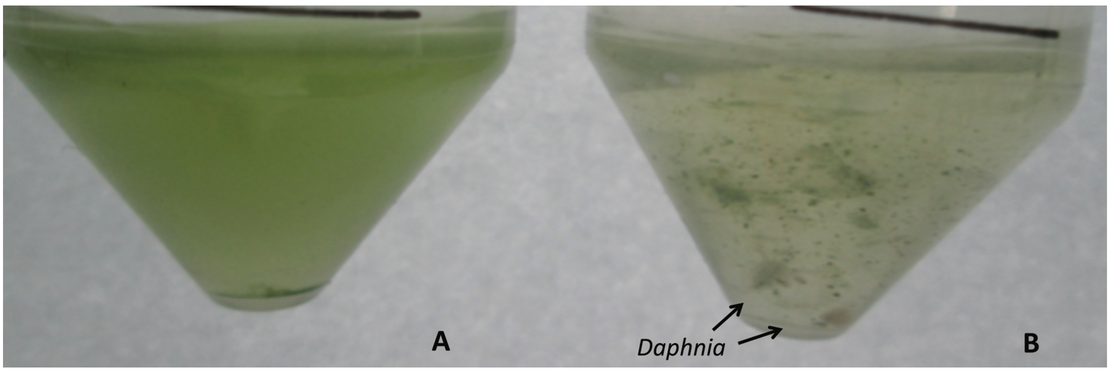


Fig. 2.9. Group formation in *C. sorokiniana* upon predation by *D. magna*. Cultures of

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).

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. 2.2), and groups being composed of larger numbers of cells in seven combinations (Fig. 2.3); (2) the presence of predator products induced algal group formation in three combinations (Fig. 2.6); (3) the presence of predators resulted in a decrease in algal density in five combinations (Fig. 2.7), and behavioural observations consistent with predation, in eight combinations (Fig. 2.8).

Response to predators

In all nine combinations, the addition of predators led to a higher proportion of cells in groups (Fig. 2.2), and in seven combinations, predators led to the formation of larger groups (Figs. 2.3 and 2.4). 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. 2.9). 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, possibly because optimal group size depends upon the type of predator (Figs. 2.2 and 2.3). 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. 2.6a.E), *C. sorokiniana* with *D. magna* (Fig. 2.6a.G), and *S. obliquus* with *D. magna* (Fig. 2.6b.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. 2.6a.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. 2.6b.I), confirming Lampert and colleagues' finding, but no group formation in *C. sorokiniana* (Fig. 2.6b.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* (Lurling 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. 2.2I), 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. 2.7A, B, G–I). We did not observe decreased algal density in four combinations: *S. obliquus* with *Ochromonas* spp. (Fig. 2.7C), *C. sorokiniana* with *T. thermophila* (Fig. 2.7D), *C. vulgaris* with *T. thermophila* (Fig. 2.7E), and *S. obliquus* with *T. thermophila* (Fig. 2.7F). 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. 2.7C–E, 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. 2.8A, B, D, F–I) suggested that in eight combinations the predators were eating the algae. Specifically, *Ochromonas* spp. captured *C. sorokiniana* (Fig. 2.8A) and *C. vulgaris* (Fig. 2.8B) 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. 2.8D), *C. vulgaris* (Fig. 2.8E), and *S. obliquus* (Fig. 2.8F), 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. 2.8G–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).

Acknowledgements

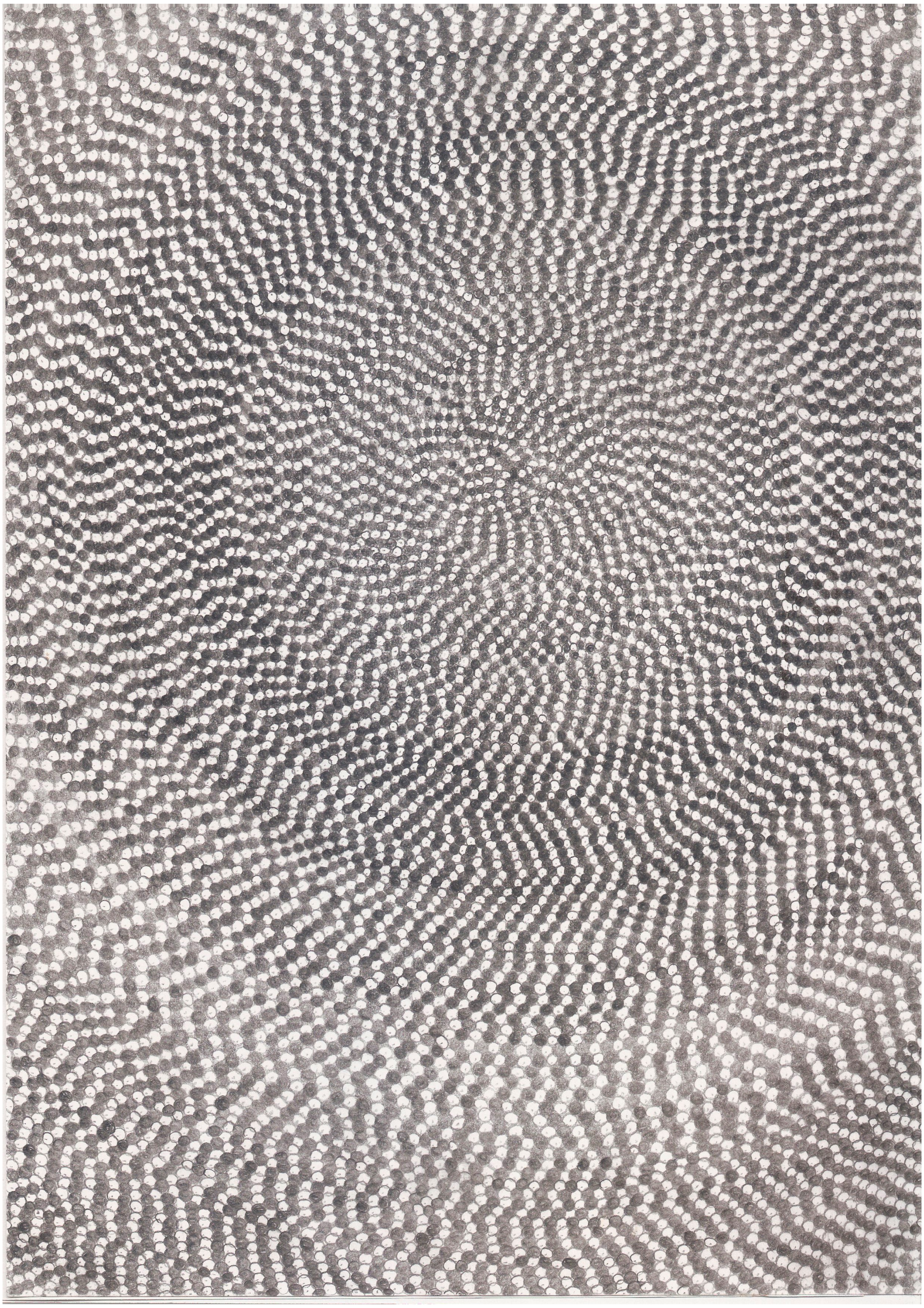
We thank Ville Friman, Tom Bell, and Lorenzo Santorelli for supplying organisms; and Andrew Beckerman, Tom Bell, Max Burton, Melanie Ghoul, Ashleigh Griffin, Karl Heilbron, Nella Roccuzzo, Matteo Tanadini, and Lindsay Turnbull for useful comments. This work was supported by the European Research Council (to S.A.W.), the Natural Environment Research Council (to R.M.F.), and the State Scholarships Foundation of Greece, the Alexander S. Onassis Public Benefit Foundation Scholarship, and A.G. Leventis Foundation Scholarship (to S.E.K.).

Data Accessibility

The experimental data for this study are freely available on Dryad:

http://datadryad.org/resource/ doi:10.5061/dryad.78nq4.

Chapter 3: How do algae form multicellular groups?*



* Published as Kapsetaki, S. E., Tep A., and West S. A. "How do algae form multicellular groups?" *Evolutionary Ecology Research* 18.6 (2017): 663-675 (appended).

Abstract

Background: Theory suggests that how groups are formed can be a major 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.

Organisms: The freshwater algae *Chlorella sorokiniana, Chlorella vulgaris* and *Scenedesmus obliquus*, and the freshwater crustacean predator *Daphnia magna.*

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: (i) emphasises the likelihood of direct fitness benefits of forming groups to avoid predation; and (ii) strengthens the across species correlation between the method of group formation and whether multicellularity is facultative or obligate.

Keywords: predation, Chlorophyceae, induced defense, 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; Grosberg and Strathmann 2007; Bourke 2011; Fisher et al. 2013). All 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* & *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 used 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 Bolds Basal media at 20^{o}C in a light/dark cycle of 16:8 hours 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 Bolds 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, light/dark cycle of 16:8 hours fluorescent illumination, and a temperature of 20^{o}C before using these cultures in experiments.

As predators, we used *Daphnia magna* (Sciento, UK), which we fed 5 mL *S. obliquus* (10^6 cells • mL^{-1}) every 4-5 days. We maintained the *Daphnia* in 500-mL jars at 20^{o}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^{TM} Fluorescent Probes). We centrifuged the exponentially growing *C. sorokiniana* at 100 x 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 x g for 10 minutes and resuspended in Bolds Basal media to remove the dyes.

We sonicated the two algal cultures (10 by 1 second pulses, amplitude 20%) to break up any groups that may have formed during the dyeing process, diluted both cultures to 10^6 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 Bolds 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 3 individuals per mL). 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 can 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 randomised the tubes on a rack in an incubator at 20^{o}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 9 images per replicate (9x3=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 3-dimensional group, especially in large groups, was difficult to determine from the 2-dimensional images (e.g. Fig. 3.1), as many cells were 'hidden in the background'. We counted what we saw in the 2-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 these time points (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.

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 3 individuals per mL) in a 50-mL falcon tube. We maintained the tube at 20^{o}C with 16:8 hours light:dark cycle with fluorescent illumination. After 10 hours, we diluted the culture using Bolds Basal media to a final concentration of 4 x 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, magnification 20x, 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” (Version 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 instance 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 ≥ 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. Out of these 50 cells, we measured the proportion that joined their group by aggregation. The remaining proportion of cells joined 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, version 3.2.3. To compare the proportion of cells in monochromatic groups between the media, predator supernatant and live predators treatments in the fluorescence experiments, we used generalised 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 with that expected from a binomial distribution. The observed variance (V_o) is given by: $s^2(1 - r^2)$, where s^2 is variance in the number of green cells per group, and r 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: $\alpha p(1\text{-}p)$, where a is group size, and p the expected proportion of green cells. Specifically p is: $(b + r\alpha)/\alpha$, 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 $\chi^2 = (V_o/V_e)/(N\text{-}2)$, 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).

Results

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 (Fig. 3.2; glm, media vs. predator supernatant & live predators, *C. sorokiniana*: $F = 131.29$, $P < 0.001$, $df = 7$, *C. vulgaris*: $F = 82.07$, $P < 0.001$, $df = 7$, *S. obliquus*: $F = 8.79$, $P = 0.02$, $df = 7$). The statistical analysis for each treatment pair is presented in Table S3.1.

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. 3.2). 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 (Fig. 3.2; 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 & 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).

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 3.1). 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 out 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 had joined their group by aggregation when exposed to predator supernatant, respectively. 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, though 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 ± 20.6 hours (mean ± s.e.m.), 10.8 ± 2.6 hours, and 51.6 ± 4.4 hours with their parent cell after division, respectively.

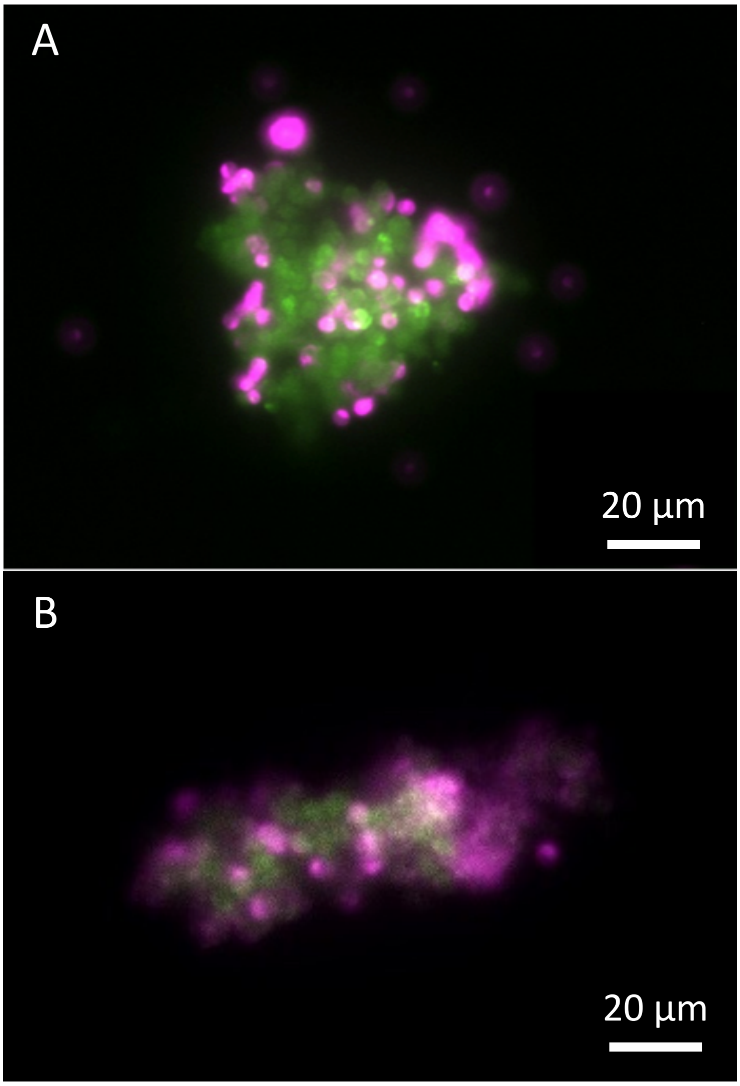


Fig. 3.1. Representative images of dichromatic groups within (A) and between (B) species. (A) Green- and violet-dyed *Chlorella sorokiniana* form a dichromatic group in the presence of *Daphnia*. (B) Green-dyed *Chlorella sorokiniana* and violet-dyed *Chlorella vulgaris* form a mixed-species dichromatic group in the presence of *Daphnia.*

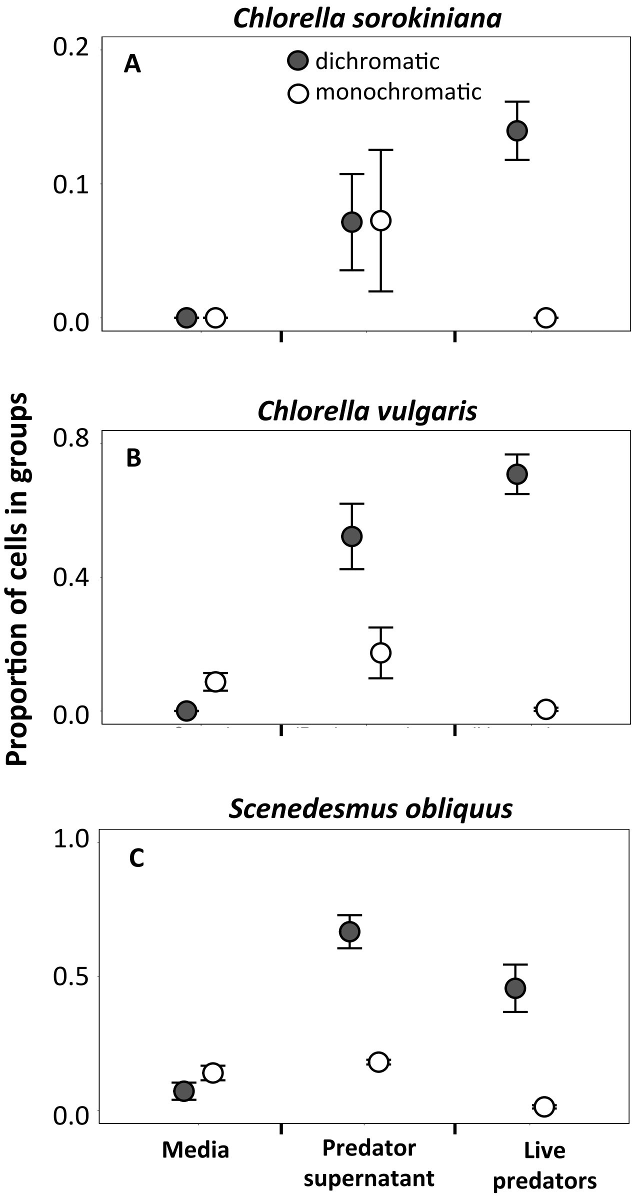


Fig. 3.2. Within-species group formation. The proportion of cells in groups is plotted in the absence of predators (media), the presence of predator supernatant and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), 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 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.

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 (Fig. 3.3; glm, media vs. predator supernatant & 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*: $F = 57.21$, $P < 0.001$, $df = 7$).

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. 3.3). 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 (Fig. 3.3; glm across the three treatments, *C. sorokiniana* with *C. vulgaris*: $F = 11.10$, $P = 0.009$, $df = 6$, *C. sorokiniana* with *S. obliquus*: $F = 15.19$, $P = 0.004$, $df = 6$, *C. vulgaris* with *S. obliquus*: $F = 36.90$, $P < 0.001$, $df = 6$; glm, media vs. predator supernatant & live predators, *C. sorokiniana* with *C. vulgaris*: $F = 23.88$, $P = 0.001$, $df = 7$, *C. sorokiniana* with *S. obliquus*: $F = 27.42$, $P = 0.001$, $df = 7$, *C. vulgaris* with *S. obliquus*: $F = 67.91$, $P < 0.001$, $df = 7$).

Additionally, in all three algal species combinations the distribution of green cells in groups showed significantly more than binomial variation when exposed to predator supernatant or live predators (Table 3.1). This suggests a greater than random propensity to form groups with members of the same species.

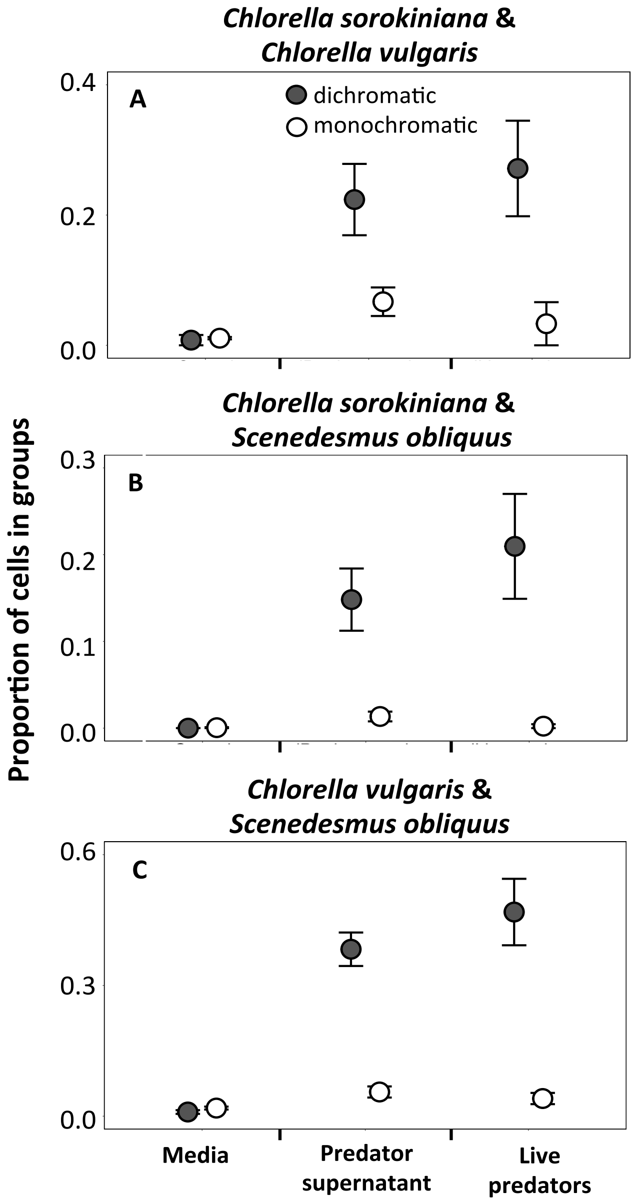


Fig. 3.3. Between-species group formation. The proportion of cells in groups is shown in the absence of predators (media), the presence of predator supernatant and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), 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. Y-axes between panels

have different values. 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.

Algae	Predators	Observed variance/ Expected variance	Number of groups sampled	χ^2-value	*P*-value
Within species					
Chlorella sorokiniana	supernatant	4.81	4	9.62	0.008
	live	1.63	3	1.63	0.201
Chlorella vulgaris	supernatant	7.49	36	254.71	< 0.001
	live	68.20	6	272.81	< 0.001
Scenedesmus obliquus	supernatant	23.75	221	5201.83	< 0.001
	live	55.57	54	2889.77	< 0.001
Between species					
Chlorella sorokiniana & *Chlorella vulgaris*	supernatant	6.22	32	186.78	< 0.001
	live	17.90	12	179.03	< 0.001
Chlorella sorokiniana & *Scenedesmus obliquus*	supernatant	17.48	24	384.61	< 0.001
	live	24.00	28	624.07	< 0.001
Chlorella vulgaris & *Scenedesmus obliquus*	supernatant	66.78	163	10752.68	< 0.001
	live	39.29	58	2200.58	< 0.001

Table 3.1. Comparison of the proportion of green-dyed cells in groups relative to a random binomial variance. We show analyses for when the different coloured cells (green & violet) are the same species, or different species, and when group formation was induced by either 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.

Discussion

We found group formation in all three algal species, *C. sorokiniana, C. vulgaris* and *S. obliquus*, in response to live predators/predator supernatant (Fig. 3.2), consistent with previous results from 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. 3.1A & 3.2). 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 3.1). 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 3.1). 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. 3.1B & 3.3).

Previous studies have suggested the necessity of cell division for group formation (Lampert et al. 1994; Trainor 1998). *C. 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 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. 3.1 & 3.2). Our results do not exclude the possibility that some group formation is via remaining with parents, because group formation is not purely random (Table 3.1), and with time-lapse photography, we observed some cells forming groups by division. Although we could not identify whether this was just division, as part of their life cycle or actual group formation (e.g. Movie S3.1). 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), though these two treatments were not conducted at the same time. 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 (J.T. 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 randomly move in the liquid culture, consistent with Brownian motion. In the presence of just predator supernatant 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 (Movie S3.2 & S3.3).

We found that not only did algae form groups via aggregation, but they also grouped with other species (Fig. 3.3). 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, Betaproteobacteria, Gammaproteobacteria 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 (Kaushik et al. 2006; Sathe et al. 2010; Sathe et al. 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 3.1). 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 (Lurling 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).

To conclude, 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; Fisher et al. 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. 2013). Consequently, our result 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).

Acknowledgements

We thank Roni McGowan, Lindsay Turnbull and Nick Harberd for providing temporary lab space, Kevin Foster for allowing us to use the fluorescent stereoscope, Isabel Frost and Jamie Wheeler for valuable assistance in using the fluorescent stereoscope, and Roberta Fisher and David Crosse for helpful comments on the manuscript. This research was funded by the European Research Council (to S.A.W.), the HSBC (to A.T.), the Alexander S. Onassis Public Benefit Foundation and the A.G. Leventis Foundation (to S.E.K.).

Appendix

Movie S3.1. (https://youtu.be/ftUyxO5hEw4) A single cell of *Scenedesmus obliquus* divides, forming a group of seven cells, in the presence of supernatant from a culture of *Daphnia magna*. An example of cell division where we cannot distinguish whether this is simply division as part of the alga's normal life cycle or actual group formation. This movie is a short section from our time-lapse experiment. Each second of the movie represents 4 minutes in real time.

Movie S3.2. (https://youtu.be/8Mf8i_jLwEI) This movie shows a cell of *C. vulgaris* dividing with the daughters sticking to the parent cell (red arrow: 1:54) and other cells dividing with the daughter cells dispersing (black arrows at 0:46, 1:12, 1:33, 1:42, 6:03, 6:12, 6:17). The algal culture is exposed to supernatant from a culture of *Daphnia magna*. This movie is a short section from our time-lapse experiment. Each second represents 4 minutes in real time.

Movie S3.3. (https://youtu.be/4F6ylfWXEks) This movie shows aggregation of cells (blue arrows at 0:08, 2:51, 3:43, 4:19, 6:50), cell division with daughter cells dispersing (black arrows at 4:26, 8:31), and daughter cell attachment after division (red arrow at 8:41). The movie is a short section from our time-lapse experiment with *Chlorella sorokiniana*. The algae are exposed to supernatant from a culture of *Daphnia magna*. Each second represents 4 minutes in real time.

	Proportion of cells in groups				Proportion of cells in dichromatic groups			
	overall comparison across treatments	media vs. predator supernatant	media vs. live predators	predator supernatant vs. live predators	overall comparison across treatments	media vs. predator supernatant	media vs. live predators	predator supernatant vs. live predators
Chlorella sorokiniana	< 0.001*	0.001*	0.002*	0.98	0.006*	0.17	0.01*	0.19
Chlorella vulgaris	< 0.001*	< 0.001*	< 0.001*	0.97	< 0.001*	0.003*	< 0.001*	0.19
Scenedesmus obliquus	0.002*	0.001*	0.07	0.01*	0.002*	0.001*	0.01*	0.13

Table S3.1. Statistical analysis of the proportion of cells in groups and the proportion of cells in dichromatic groups across treatments, in the within-species fluorescent experiments of *C. sorokiniana*, *C. vulgaris* and *S. obliquus*. The "overall comparison across treatments" columns show *P*-values from generalised linear models across the three treatments (media, predator supernatant, live predators), using the quasibinomial family to account for overdispersion. The remaining columns show *P*-values from Tukey post-hoc tests testing for significant differences between treatment pairs. Asterisks indicate significant values ($P < 0.05$).

Time-lapse experiment of algae in Bolds Basal media

Materials & Methods

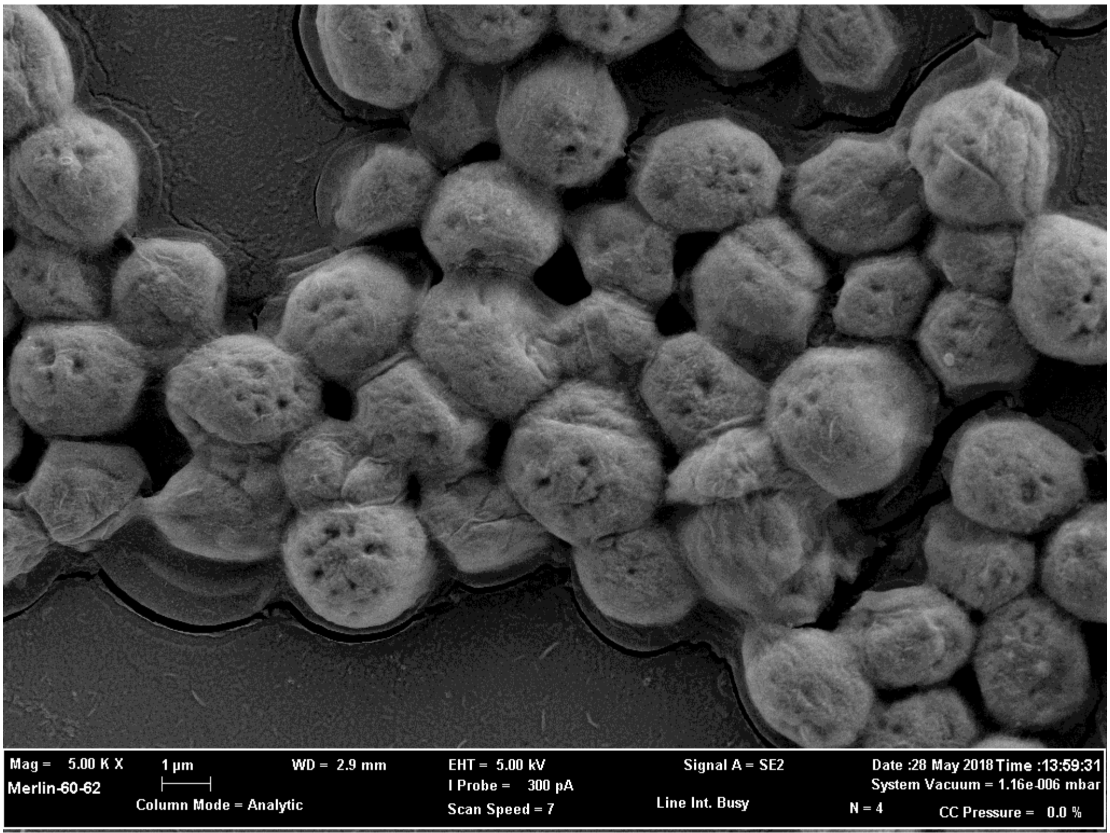
We tested how *C. sorokiniana* forms groups in the absence of predators using time-lapse photography. We added 4.04 mL of *C. sorokiniana* (initial concentration of 10^6 cells $\cdot$ mL^{-1}) to 0.96 mL of filtered Bolds Basal media in a 50-mL falcon tube. We maintained the tube at 20°C with 16:8 hours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

From the end of the movie, we randomly chose a cell in a group and tracked it back in time, stopping at the first instance at which it joined this group. We observed 30 cells, each from a different randomly selected group that had joined their group as a result of division from their parent cell, and measured the time they spent with their parent cell after division. We followed the same experimental procedure for *C. vulgaris* and *S. obliquus.*

Results

Out of the 30 cells we tracked, we found that in the absence of predators, the average time daughter cells spent with their parent cell was 2.0 ± 1.3 hours (mean ± s.e.m.) in *C. sorokiniana*, 5.2 ± 2.1 hours in *C. vulgaris,* and 38.0 ± 4.2 hours in *S. obliquus*.

Chapter 4: The costs and benefits of multicellular group formation in algae*



Our ability as humans to quickly identify human faces led me to notice a cell in the group displaying a 'human-like happy face' (image courtesy: Koen Evers). It looks like someone's happy to be in a clump (!), while others look rather grumpy.

So let's look at the benefits and costs of clumping…

* Submitted at *Evolution* as: Kapsetaki S.E., West S.A. "The costs and benefits of multicellular group formation in algae".

Abstract

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 the formation of multicellular groups. Green algae offer excellent opportunities for studying multicellular group formation, because group formation is facultative, and can be experimentally induced. This allows group formation to be experimentally manipulated independent of its potential benefits and costs. We found that in the 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, multicellular groups break up at a rate that does not vary with light intensity. A meta-analysis of four different algal species revealed that the benefit of group formation is higher when algae are exposed to smaller predators, and the cost of group formation is significantly higher under resource limiting conditions. Our results show that multicellular group formation can be favored to avoid small predators, but that multicellular group formation can also lead to growth costs, explaining why multicellular group formation is facultative, in response to the presence of predators.

Keywords: predation, major evolutionary transitions, cooperation, multicellularity, *Chlorella*

Introduction

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 is that forming groups provides a defense against relatively small predators, but not against relatively large predators that can consume groups. If group formation has costs, then this could help explain why group formation is only facultative, in response to predators. 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 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 four 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: (i) both species predate *C. sorokiniana* sufficienctly to impact population density, and (ii) *O. danica* is approximately 100 times smaller than *D. magna*, allowing us to examine whether group formation provides a benefit against a small and a large predator (Kapsetaki et al. 2016). *O. danica* like many flagellate and ciliate predators is of similar size to algal cells whereas the larger predator *D. magna* can be much larger than groups, and so predation could potentially be more impaired by group formation in the case of *O. danica* (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 favoured 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).

Fourth, we performed two meta-analyses including our data, testing whether the benefit of group formation is higher upon exposure to small predators, and whether the cost of group formation is higher when resources are limited.

Methods

Strains

We maintained the algae *Chlorella sorokiniana* (non-axenic from the Culture Collection of Algae and Protozoa; strain number 211/8K) in Bolds Basal media at 20^{o}C at a light:dark cycle of 16:8 hours fluorescent illumination. 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 hours in Bolds Basal media (Kapsetaki et al. 2016; Kapsetaki et al. 2017). We maintained the cultures in 1-L Erlenmeyer flasks shaking at 220 rpm, at 20^{o}C and a light:dark cycle of 16:8 hours fluorescent illumination, before using the cultures in experiments.

As putative predators, we used *Ochromonas danica* (axenic from the National Center for Marine Algae and Microbiota; strain number CCMP 3279) cultured in 2.5 mL Proteose Peptone Yeast extract (PPY) media diluted in 7.5 mL dH_2O, and *Daphnia magna* (Sciento, UK), which we fed 5 mL *Scenedesmus obliquus* (10^6 cells mL^{-1}) every 4-5 days. We maintained the *O. danica* culture in a 50-mL Falcon tube and the *D. magna* in 500-mL jars and at 20^{o}C and a light:dark cycle of 16:8 hours 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 minimise 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 x 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 3 individuals per mL) 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^{o}C and a light:dark cycle of 16:8 hours fluorescent illumination. We kept the tube caps loose to allow oxygenation. We collected samples at 0 and 24 hours by tilting each tube 5 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) at x20 magnification. In these 2-dimensional images, we quantified the proportion of cells in groups (number of algal cells in groups/total number of algal cells). We define a group as ≥3 cells in contact with each other, but found the same qualitative results when analysing mean group size. In several cases the exact number of cells in 3-dimensional groups, especially in large groups, was difficult to determine from the 2-dimensional images, as many cells were ‘hidden in the background’. For consistency, we counted what we saw in the 2-dimensional images. We counted paired cells as single cells in all the experiments.

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 3 individuals per mL) (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^{o}C at 16:8 hours light:dark cycle fluorescent illumination for 96 hours. We mixed replicates of the same treatments resulting in two cultures, and diluted both cultures to 40 algal cells per field of view at x20 magnification. We then created four experimental treatments in a 2x2 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^{o}C and a light:dark cycle of 16:8 hours. After adding the predator treatments, we collected samples at 0 and 3 hours, 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 hours after adding predators - live *O. danica* induces group formation in *C. sorokiniana* after 24 hours, but not after 3 hours (see results; 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 3 individuals per mL) (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^{o}C at 16:8 hours light:dark cycle fluorescent illumination for 96 hours. 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^{o}C and a light:dark cycle of 16:8 hours. After adding the predator treatments, we collected samples at 0 and 12 hours, 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 3 individuals per mL) (6 replicates per treatment). We kept the falcon tube caps loose to allow for gas exchange and randomized tubes on a rack in an incubator at 20^{o}C with a light/dark cycle of 16:8 hours using fluorescent illumination.

After 48 hours, 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 x g for 10 minutes and resuspended the pellet in CD-CHO Medium (Gibco, Carlsbad, CA). We then split each 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 four cultures with aluminium foil, left them shaking at 170 rpm overnight at room temperature, then centrifuged both cultures at 100 x g for 10 minutes, 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 3 medium-sized *D. magna*; (2) 1 mL of the multicellular violet:unicellular green culture and 3 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 hours, constructed fluid tunnel slides (Kapsetaki et al. 2017), and imaged samples under a Zeiss Axio Zoom V16 fluorescence stereoscope (Carl Zeiss, Oberkochen, Germany) at x100 magnification. We took 3 images per replicate and measured the number of green and violet cells. We averaged these 3 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. 4.4A), which reduced light and subsequent algal growth (see 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. 4.4A); (3) 0.96 mL of filtered liquid from the culture of *D. magna* (final concentration of 3 individuals per mL) 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 5 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 hours fluorescent illumination, keeping the tube caps loose to allow for oxygenation. After adding the predator treatments, we collected samples at 0 and 96 hours, using the abovementioned sampling protocol, and measured the total number of algal cells and the proportion of cells in groups.

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 aluminium foil from the bottom of the tubes creating a hole through which light could pass (see Fig. 4.5A). 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 3 individuals per mL) (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 an incubator at 20^{o}C with a light:dark cycle of 16:8 hours using fluorescent illumination. On day 4, we covered the 18 tubes which had been treated with *D. magna* supernatant in aluminium foil (see Fig. 4.4A), and left the remaining 18 tubes without aluminium foil. After adding the *D. magna* supernatant we obtained samples on day 0 (0 hours), 1 (24 hours), 4, 7, 10, and 13, 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, $F_{3,32} = 0.07$, $P = 0.97$).

Meta-analyses

We assessed whether the benefit of algal group formation, i.e. multicellular algae having higher survival rates than unicellular algae, is higher when exposed to smaller predators (‘benefit meta-analysis’); and whether the cost of algal group formation, i.e. multicellular algae having lower growth rates than unicellular algae, is higher under resource-limiting conditions (‘cost meta-analysis’).

We collected data by searching on Google Scholar and Web of Science using the following keywords, "alga*” OR “microalga*” OR “*Chlorophyceae” OR “Chlorella” OR “Scenedesmus”* AND “coenobi*” OR "multicellular group*” OR "group formation" OR "colon*” OR “clump*” OR "colony formation" OR “multicell*” OR “aggregate” OR “flocculation” OR “floc*” OR “mean particle volume” AND “predator*” OR “graz*” "benefi*” OR "cost*” OR "grazing resistance*” OR “sink*”, and searched studies backwards and forwards without constraint on publication year. This search led to a total of ~1830 studies. We removed duplicate and ineligible studies (see Fig. S4.2, Table S4.1). We collected data on mean number of algae, mean algal growth or clearance rate, standard deviations, sample size, and predator size or resource availability (high and low levels of light or nutrients). When studies did not provide means or standard deviations in the text, we obtained these manually from figures using the software WebPlotDigitizer. For the ‘benefit meta-analysis’ we included studies that compared clearance/growth rate values of unicellular and multicellular algal cultures upon predation, and for the ‘cost meta-analysis’ we included studies that compared growth values of unicellular and multicellular algal cultures under high and low resources. We only used studies where we had data available for two or more predator sizes/resource availability per algal species. For the ‘benefit meta-analysis’ we used three studies (including this study) of 22 effect sizes in total and four algal species, and for the ‘cost meta-analysis’ we used seven studies (including this study) of 22 effect sizes in total and four algal species.

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 analysing 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 analysis which we did not carry out with a generalized linear model, was when examining the effect of *Daphnia* size (large, small, 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, since that is when the tubes were separated into light and dark conditions.

For the meta-analyses, we analyzed data using the multivariate meta-analysis model ('rma' function) in the R package 'metafor' (Viechtbauer 2010). In the 'cost meta-analysis' we categorized data on a scale of low (0) and high (1) resource availability. For the 'benefit meta-analysis', we measured the average size (μm) of every predator species and normalized these data by measuring the square root per average predator size. We built separate regressions for each species treating resource availability ('cost meta-analysis') or the square root of average predator size ('benefit meta-analysis') as a fixed effect in our model. We determined whether the mean slope across the four species was different from zero by building a regression ('rma' function) and treating resource availability*species ('cost meta-analysis') or the square root of average predator size*species ('benefit meta-analysis') as fixed effects in our model. As effect sizes we used the natural log-transformed ratios of the mean number of algae/algal growth rate in unicellular cultures divided by the mean number of algae/algal growth rate in multicellular cultures ('cost meta-analysis'); and the natural log-transformed ratios of the mean clearance rate of algae in unicellular cultures divided by the mean clearance rate of algae in multicellular cultures when studies only provided values of clearance rates, or the mean total number of algae in multicellular cultures divided by the mean total number of algae in unicellular cultures when values of total algae were provided ('benefit meta-analysis'). Using the effect size as a standardized scale to compare data across studies allows us to see the overall pattern even though there may be differences in experimental techniques between studies. We measured variances using Hedges' et al. (1999) variance equation [V = s1^2/(n1*Y1^2) + s2^2/(n2*Y2^2), where V = variance; n1, n2 = sample sizes of each treatment; s1, s2 = standard deviations of each treatment; Y1, Y2 = means of each treatment].

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. 4.1; 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. 4.1A). 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.

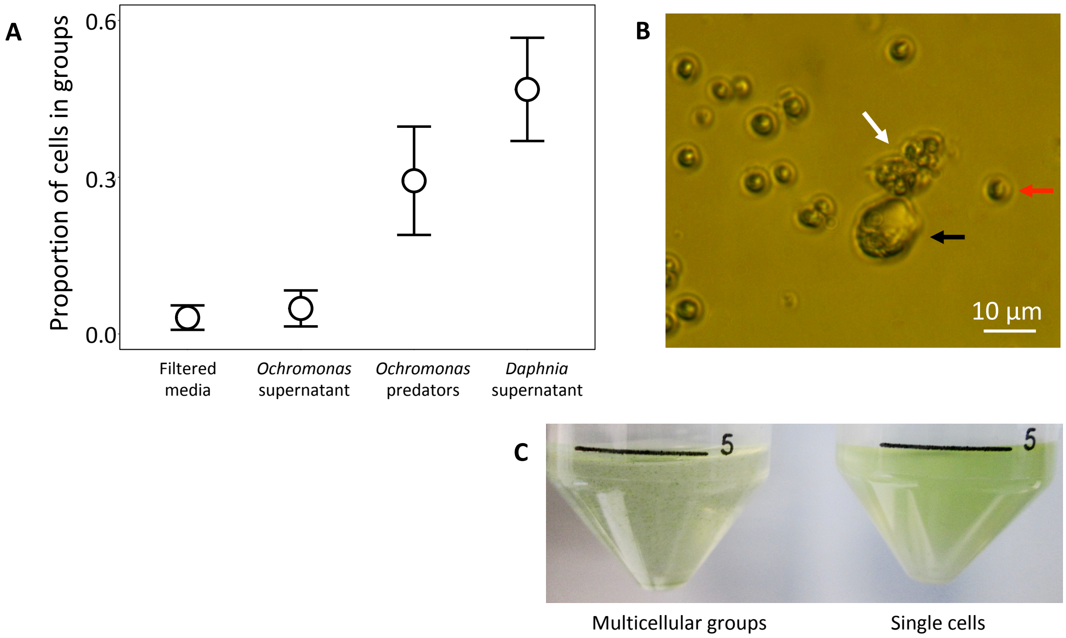


Figure 4.1. Group formation in the algae *C. sorokiniana*. (**A**) The addition of both live *O. danica* predators and *D. magna* supernatant, but not the addition of *O. danica* supernatant, led to an increase in the proportion of cells in multicellular groups. These results are in comparison to our control treatment, where we added filtered media. Error bars show 95% confidence intervals (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 hours 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.

Ochromonas predator

We found that the formation of multicellular groups in the algae *C. sorokiniana* reduced predation by *O. danica* (Fig. 4.2). 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). 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. 4.2B).

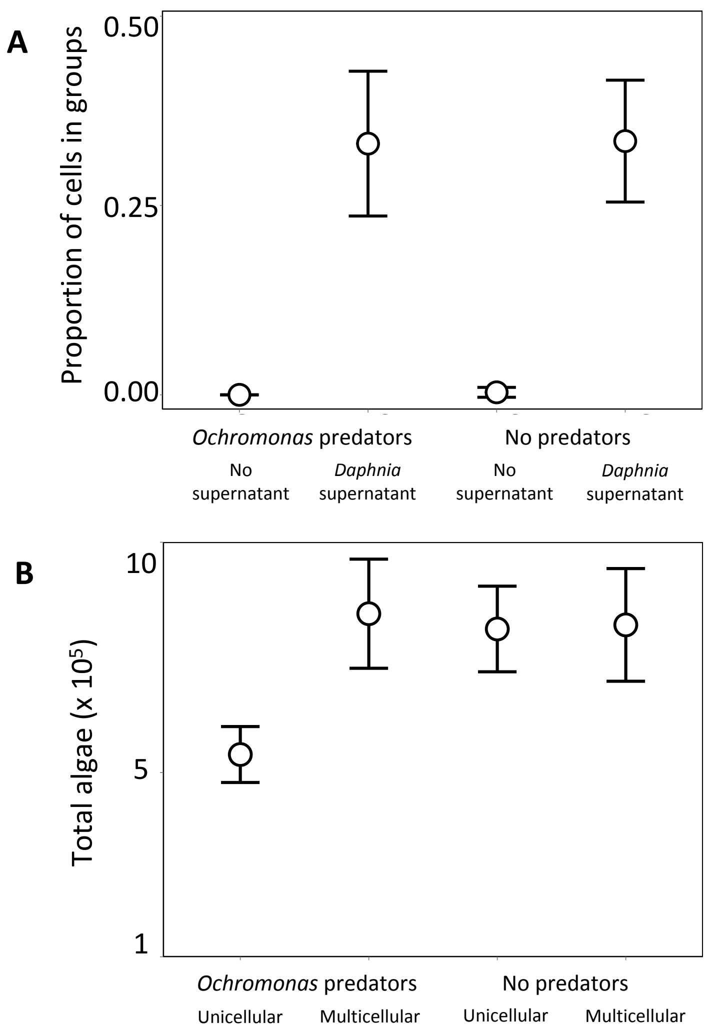


Figure 4.2. The benefit of multicellular group formation in the algae *C. sorokiniana* upon predation by *O. danica*. (**A**) The proportion of algal cells in multicellular groups was manipulated by adding *D. magna* supernatant. The addition of *D. magna* supernatant, prior to adding *O. danica* predators, led to a significantly higher proportion of cells in multicellular groups, as also shown in Figure 4.1. In contrast, the proportion of cells in multicellular groups did not change significantly in response to three hours 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% confidence intervals (30 independent replicates per treatment).

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. 4.3). 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. 4.3A). 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. 4.3B). The reduction in number of algae (predation) was greater with larger *D. magna* (Fig. 4.3B; linear model, adjusted $r^2 = 0.38$, $F_{1,106} = 68.71$, $P < 0.0001$). Third, in multicellular (dyed green or violet):unicellular (dyed violet or green) algal cultures, the 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. 4.3C). In both cultures, medium-sized *D. magna* decreased total algal density after 3 hours ($F_{1,69}$ = 15.39, P = 0.0002), indicating predation.

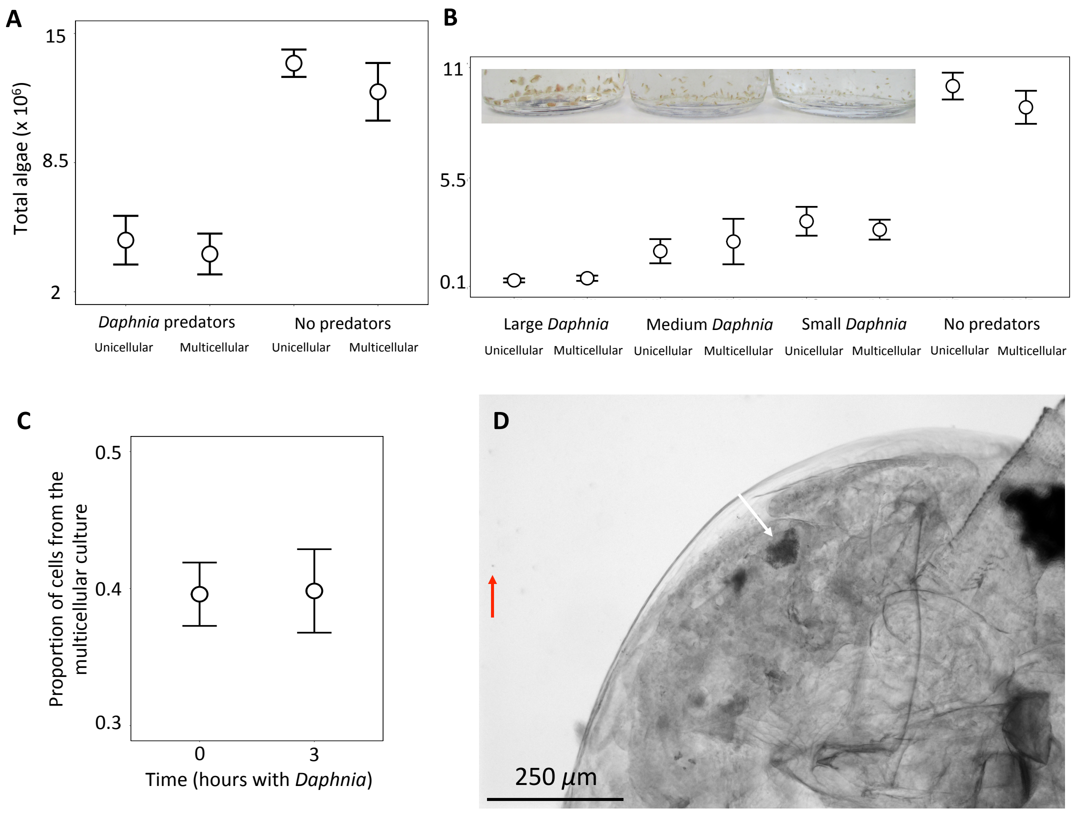


Figure 4.3. Multicellular group formation does not reduce predation by *D. magna*. (**A**) The rate at which *D. magna* predators predated algae did not vary significantly dependent upon whether the algae were in multicellular groups. (**B**) Across different sized *D. magna* predators, the rate at which 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% confidence intervals (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.

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. 4.4B; $F_{1,18} = 269.28$, $P < 0.0001$).

We found that whether populations were maintained under light or dark conditions did not influence the proportion of cells growing in multicellular groups (Fig. 4.4B; $F_{1,17} = 0.54$, $P = 0.46$; 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 4.4C; $F_{1,16} = 12.56$, $P = 0.002$). 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 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 aluminium foil (‘dark with hole’ treatments; Fig. 4.5A), 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. 4.5C; $F_{1,56}$ = 6.19, P = 0.01),. Algae formed larger precipitates in single-celled cultures than in multicellular cultures (Fig. S4.1), further supporting the hypothesis that the observed decrease in algal density (Fig. 4.4C) is due to multicellular groups competing for light, not due to sinking.

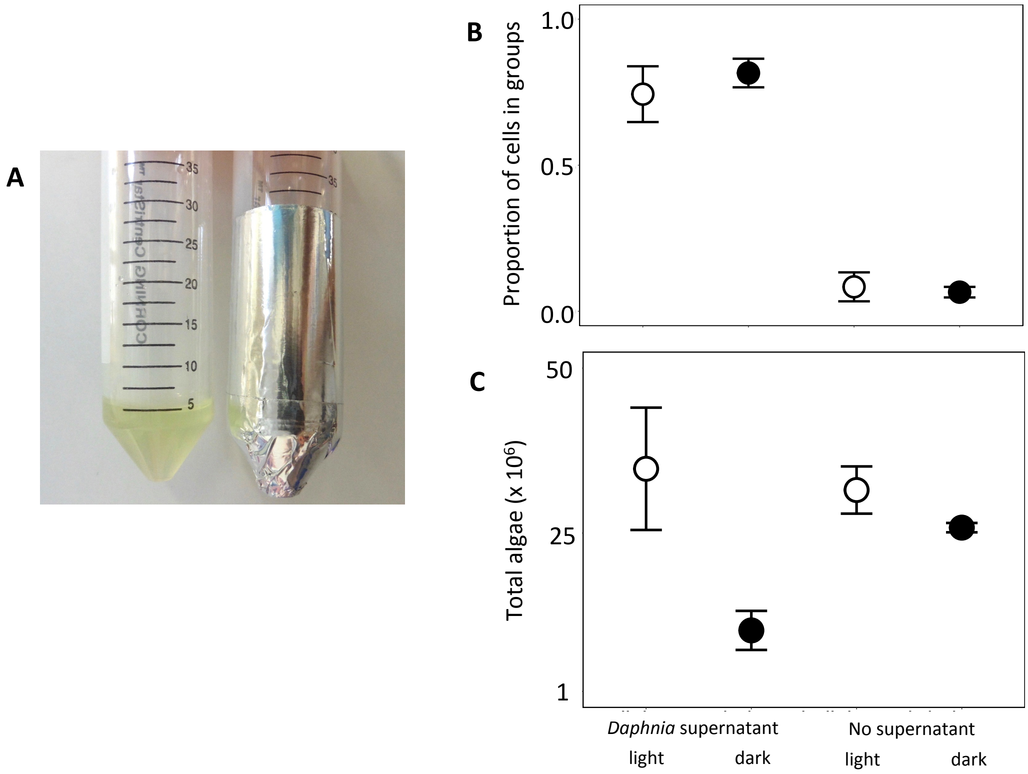


Figure 4.4. The cost of group formation in the algae *C. sorokiniana*. (**A**) We used aluminium foil to reduce the light availability. The left tube is the 'light' treatment and the right tube is the 'dark' treatment. (**B**) The addition of *D. magna* supernatant led to a higher proportion of cells 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% confidence intervals (5 independent replicates per treatment).

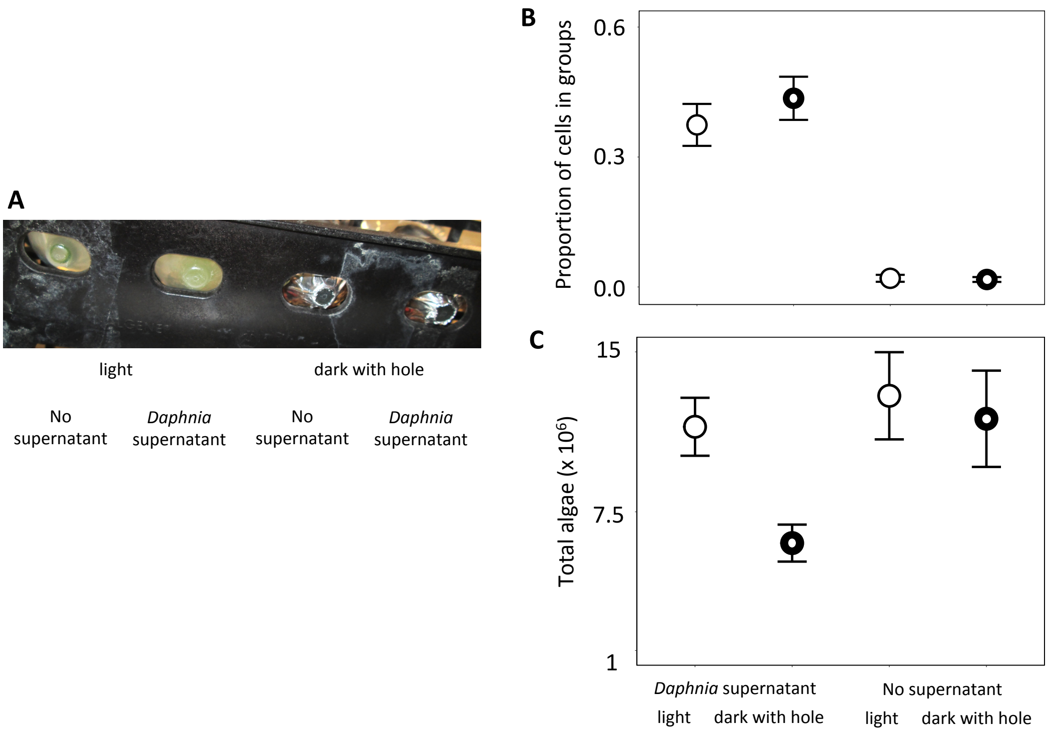


Figure 4.5. The cost of group formation is not due to sinking. (**A**) The first two cultures from the left are the 'light' treatments, and the remaining two are the 'dark with hole' treatments. In the latter, light was allowed to pass from the bottom of tubes by creating a small hole in the aluminium 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, i.e. cells restricting other cells access to light. Both plots show error bars of 95% confidence intervals (15 independent replicates per treatment).

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, groups gradually break up over time (Fig. 4.6; glmmPQL, $P < 0.0001$). The rate at which groups broke up did not vary significantly depending upon whether cells were maintained in the dark or light (Fig. 4.6; from Day 4 onwards, glmmPQL, $P = 0.47$).

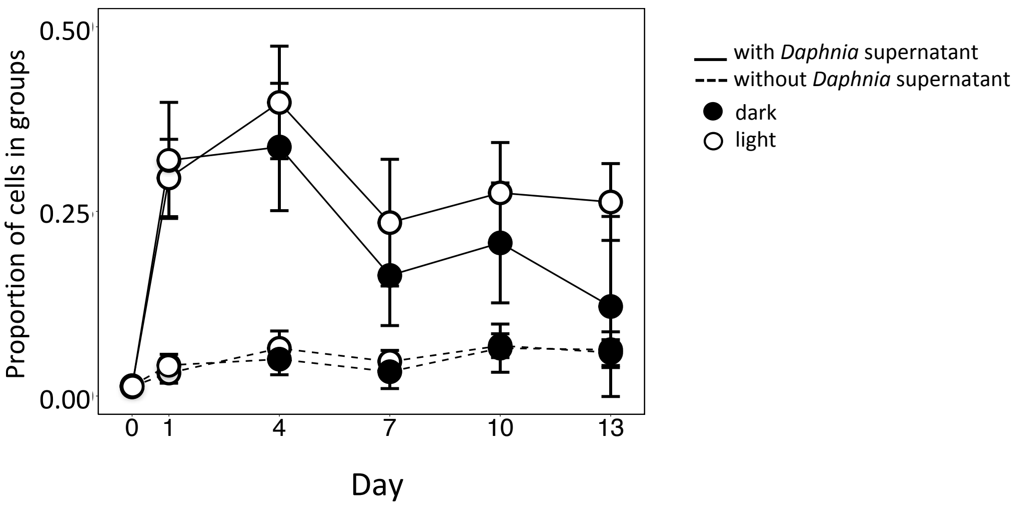


Figure 4.6. Multicellular groups of the algae *C. sorokiniana* did not break up faster in the dark than in the light. At time point 0, we exposed 50% of the 36 replicate tubes of algae to *D. magna* supernatant (solid line) leading to an increase the proportion of cells in multicellular groups. On day 4, we wrapped 50% of the tubes in aluminium foil, to produce a 'dark' treatment. The rate at which multicellular groups of algae broke up did not vary depending upon whether they were kept in light or dark conditions. Error bars show 95% confidence intervals.

Meta-analyses

By gathering data from three studies on the benefit of algal group formation and performing a meta-analysis, we found that in *C. sorokiniana*, *S. acutus*, and *S. obliquus*, but not in *S. subspicatus*, the benefit of being in a group, as expressed by the log-transformed number of multicellular/unicellular algae, is greater upon exposure to smaller predators (multivariate meta-analysis model; $slope_{C.s.} = -0.0082$, $P_{C.s.} = 0.0016$, $n_{C.s.} = 5$; $slope_{S.a.} = -0.0147$, $P_{S.a.} = 0.0059$, $n_{S.a.} = 11$; $slope_{S.o.} = -0.26$, $P_{S.o.} < 0.0001$, $n_{S.o.} = 4$; $slope_{S.s.} = -0.103$, $P_{S.s.} = 0.31$, $n_{S.s.} = 2$). Overall, across these four algal species the benefit of group formation is higher when algae were exposed to smaller predators (Fig. 4.7; multivariate meta-analysis model; slope = – 0.008, $P = 0.0016$, n = 22; the slope is significantly different than 0).

Furthermore, in *C. sorokiniana* and *Phaeocystis globosa*, but not in *S. acutus* and *S. obliquus*, we found that the cost of group formation, as expressed by the log-transformed number of unicellular/multicellular algae, is higher under limited resources (multivariate meta-analysis model; $slope_{C.s.} = -0.73$, $P_{C.s.} < 0.0001$, $n_{C.s.} = 4$; $slope_{P.g.} = -0.33$, $P_{P.g.} < 0.0001$, $n_{P.g.} = 8$; $slope_{S.a.} = 0.11$, $P_{S.a.} = 0.21$, $n_{S.a.} = 4$; $slope_{S.o.} = -0.03$, $P_{S.o.} = 0.69$, $n_{S.o.} = 6$). Overall, in these four algal species the cost of group formation is higher in resource-limiting conditions (Fig. 4.8; multivariate meta-analysis model; slope = – 0.72 ± 0.25, where 0.25 represents the 95% CI, $P < 0.001$, n = 22; the slope is significantly different than 0).

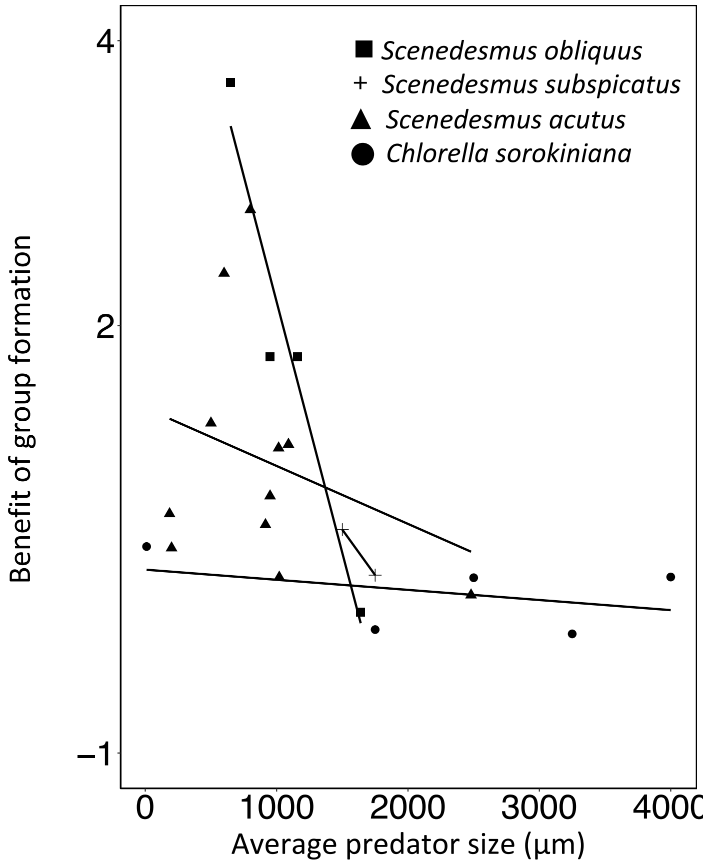


Figure 4.7. The benefit of algal group formation is higher upon exposure to smaller predators. Each data point in this meta-analysis is a different experimental study with a specific value for the benefit of group formation and average predator size (total n = 22 studies). Values on the y-axis represent the log-transformed mean number of multicellular algae/mean number of unicellular algae. Different shapes show the four different algal species.

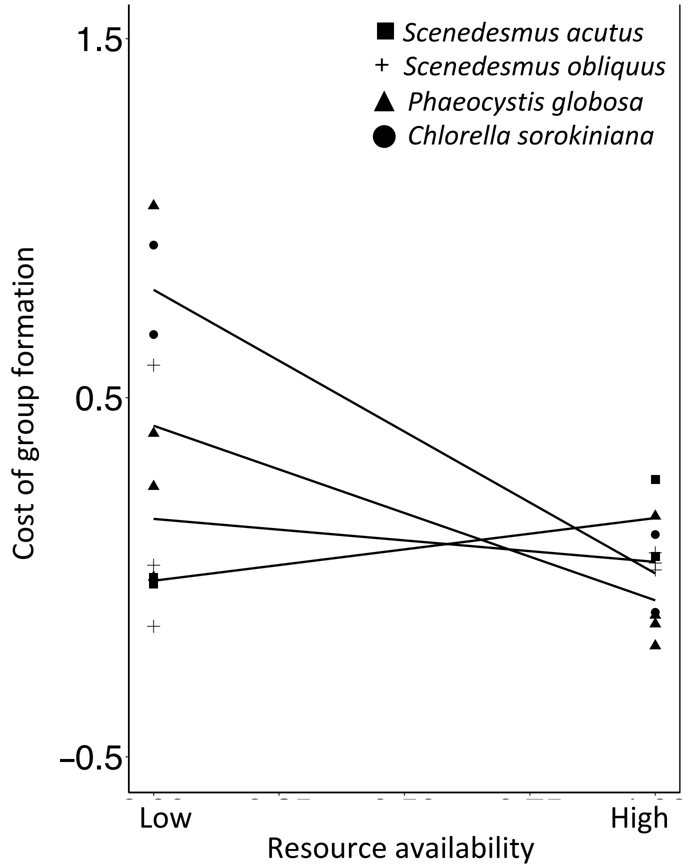


Figure 4.8. Algal group formation is more costly under limited resources. Each data point in the meta-analysis is a different experimental study with a specific value for the cost of group formation and resource availability (total n = 22 studies). Values on the y-axis represent the log-transformed mean number of unicellular algae/mean number of multicellular algae. Different shapes show the four different algal species.

Discussion

We found that in the algae *C. sorokiniana* the formation of multicellular groups can provide both a benefit in the presence of the small predator *O. danica*, but not the larger predator *D. magna*, and a cost. In particular, group formation: (1) reduces predation by *O. danica* (Fig. 4.2B); (2) doesn't reduce predation by *D. magna* (Fig. 4.3); and (3) leads to reduced algal growth under conditions of relatively low light intensity due to competition for light (Fig. 4.4C, 4.5). Over time, in the absence of predators, multicellular groups break up, but the rate of break up did not vary depending upon whether algae were grown in the dark or light (Fig. 4.6). Our meta-analyses showed that group formation in algae is more beneficial upon exposure to smaller predators, and that group formation is more costly under limited resources (Fig. 4.7, 4.8). However, both meta-analyses would certainly benefit from larger statistical power by the addition of future studies on the benefit and cost of group formation in algae.

Multicellular group formation in algae

Previous studies formally 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). Others have claimed but not formally shown 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*) (Fig. 4.2B, 4.3A, 4.3D). We confirmed that our negative result with *D. magna* was not due to a reduction of predation by only certain size *D. magna* (Fig. 4.3B) or by an effect on relative predation rates when different size groups were available (Fig. 4.3C). Our 'benefit meta-analysis' also showed that group formation is more beneficial upon exposure to smaller predators (Fig. 4.7). Out of the four species in the meta-analysis, *S. subspicatus* is the only species where the benefit of group formation is not higher upon exposure to smaller predators. However, this is the only species out of the four, know 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).

Previous studies formally 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 (Riegman et al. 1992; Peperzak 1993). 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 Harvell 1999; Tollrian and Dodson 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 (Fig. 4.4C, 4.5). This pattern was also apparent in the 'cost meta-analysis' we conducted across four different algal species revealing that algal group formation is overall more costly under limited resources (Fig. 4.8).

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. 4.6). 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). More generally, the variation across previous studies raises many questions regarding the benefits and costs of group formation. For example, does predator size affect the benefit of group formation? Our observations that groups survive predation by the small predator *O. danica*, but not the larger predator *D. magna* (Fig. 4.2B, 4.3), and our 'benefit meta-analysis', suggest that the benefit of algal group formation is higher upon exposure to smaller predators. Also, apart from group formation, algae may defend themselves against predators in many ways, including vertical migration, toxin production, thicker cell wall, spine and cyst formation (Van Donk et al. 2011).

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 moulds 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). 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 is made to obligate multicellularity, and a new higher level organism (Maynard Smith and Szathmary 1995; Bourke 2011; West et al. 2015).

Authors' contributions

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

Acknowledgements

We thank: Lindsay Turnbull and Nick Harberd for providing temporary lab space; Kevin Foster for providing access to the fluorescence stereoscope; Andrew Beckerman, Jay Biernaskie, Michael Bonsall, Michael Brockhurst, Philip Downing, Rebecca Goldberg, Roberta Fisher, and Gijsbert Werner for helpful discussion or comments. This research was funded by the European Research Council (to S.A.W.), the Alexander S. Onassis Public Benefit Foundation, and the A.G. Leventis Foundation (to S.E.K.).

Competing interests

We declare we have no competing interests.

Supplementary Material

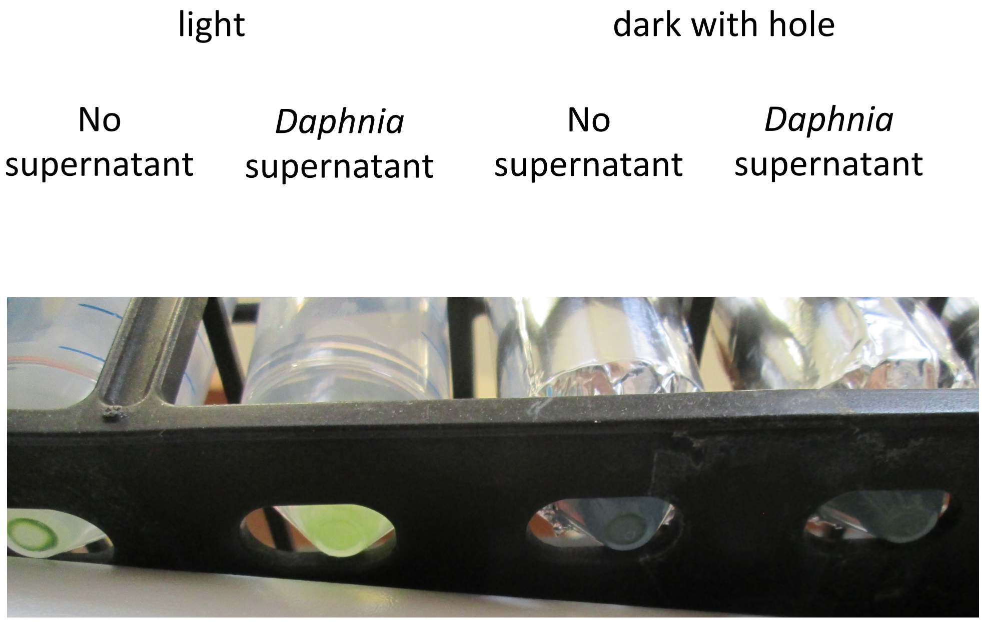


Figure S4.1. **Algae precipitate when not exposed to *Daphnia* supernatant.** The first two cultures from the left show the 'light' treatments, and the remaining two cultures show the 'dark with hole' treatments after removing the foil from the bottom of the tubes revealing the precipitate present in the treatments that have not been exposed to *D. magna* supernatant. Such a precipitate is not visible in the treatments with *D. magna* supernatant. This image shows the cultures 96 hours after exposure to either *D. magna* supernatant/no supernatant and grown in 'light'/'dark with hole', but importantly before the tubes were tilted to collect samples of which counts are shown in Figures 4.5B and 4.5C in the main text. Tilting breaks the precipitate, leaving the algae homogenised in the solution.

Figure S4.2. PRISMA Flowchat.

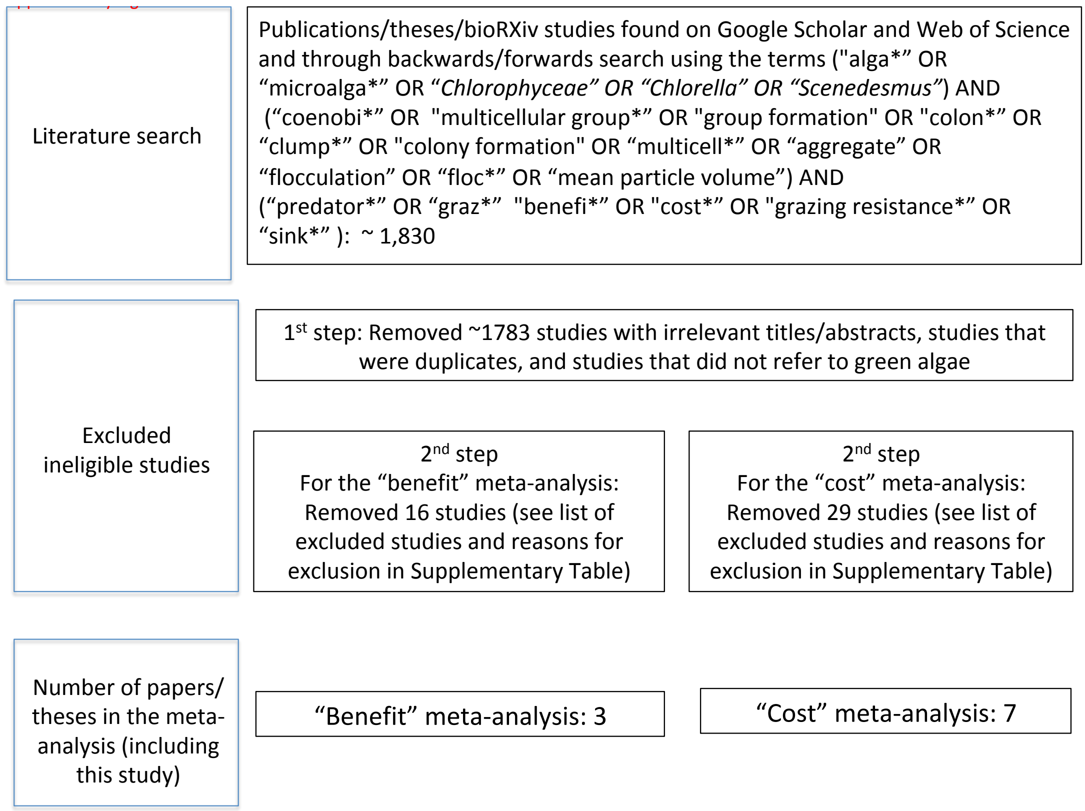


Table S4.1. Excluded studies from the meta-analysis and reason for exclusion.

Studies excluded from the meta-analyses

Benefit meta-analysis

Citation	**Organism(s)**	**Reason for exclusion**
Boraas, ME; Seale, DB; Boxhorn, JE 19	*Chlorella vulgaris* & *Ochromonas vallescia*	Did not formally measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Lampert, Winfried, Karl Otto Rothhaupt,	*Scenedesmus acutus* & *Daphnia*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Lürling, Miquel, and Ellen Van Donk. "Z	*Scenedesmus acutus* & *Daphnia*	The data in this publication are reported in Chapter 7 of Lurling's 1999 thesis, which is included in the meta-analysis.
Lürling, M. F. L. L. W. "Investigation of	*Scenedesmus pectinatus* & *Brachionus calyciflorus*	No predators have been added in the 'unicellular' treatment. Therefore we cannot examine the effect of predators on algal density in the unicellular and multicellular cultures.
Lürling, Miquel. "GRAZER-INDUCED	*Scenedesmus obliquus* & *Daphnia*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Lürling, M. F. L. L. W. "Phenotypic plast	*Scenedesmus obbliquus* & *Daphnia*	A review. Provides a summary of already published data that I include in the meta-analysis.
Lürling, M., and W. Beekman. "Grazer-i	*Scenedesmus acutus* & *Daphnia*	The data in this publication are reported in Chapter 7 of Lurling's 1999 thesis, which is included in the meta-analysis.
Lürling, Miquel, and Ellen Van Donk. "M	*Scenedesmus acutus* & various zooplankton	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Mayeli, S. M., S. Nandini, and S. S. S. S	several species of *Scenedesmus* & zooplankton	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Mulderij, G., W. M. Mooij, and E. Van D	*Scenedesmus obliquus* & *Stratiotes aloides*	*Stratiotes* is not a predator of *Scenedesmus*.
Pančić, Marina, and Thomas Kiørboe. "P	several phytoplankton & zooklankton	A review. Provides a summary of already published data that I include in the meta-analysis.
Van Donk, E. L. L. E. N., Miquel Lürling	*Scenedesmus* & *Daphnia*	The data in this publication are reported in Lurling's 1999 thesis, which is included in the meta-analysis
Von Elert, Eric, and Achim Franck. "Col	*Scenedesmus acutus* & *Daphnia*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Wu, Xinyan, et al. "Grazer density-deper	*Scenedesmus obliquus* & *Daphnia*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Zhu, Xuexia, et al. "Grazer-induced mor	*Microcystis aeruginosa* & *Scenedesmus obliquus*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Zhu, Xuexia, et al. "Response of grazer-i	*Scenedesmus obliquus* & *Daphnia magna*	Did not measure differences in algal growth between multicellular and unicellular algal cultures when exposed to predators.
Cost meta-analysis		
ACKLEH, AS; HALLAM, TG; MULLE	diatoms	The study was performed on diatoms, not green algae.
Boraas, ME; Seale, DB; Boxhorn, JE 19	*Chlorella vulgaris*	Did not formally measure growth of colonies versus single-cells in the dark.
Geng, Linlin, et al. "No difference in col	*Scenedesmus obliquus*	The 2 lake environments to which the algae were exposed differed in many aspects apart from high and low lutrient levels. For example, temperature, effective light, and physicochemical factors were different between the 2 lake treatments.
Illman, A. M., A. H. Scragg, and S. W. Sl	*Chlorella* strains	The study did not assess growth rates of single-celled algae and colonial forms under high and low nutrients.
Jakobsen, Hans H., and Kam W. Tang. "I	*Phaeocystis globosa*	There were many different nutrient media and fluorescent light conditions used across papers in the literature. Therefore, if a paper such as this only reported growth rates of single-celled and multicellular cultures in a specific nutrient media and fluorescent light condition, we were not able to classify this resource availability as "0" or "1" or place it in the continuum between 0-1. Therefore, in the cost meta-analysis, we would only include a study that compares growth rates of single-celled and multicellular cultures in different resource availability (high or low light availability/nutrients) with all other factors in the experimental comparison being equal.
KIORBOE, T; HANSEN, JLS 1993; PH	diatoms	The study was performed on diatoms, not green algae.
Li, Ming, Li Gao, and Li Lin. "Specific g	*Scenedesmus obliquus*	The study did not formally compare growth rates between single-celled algal cultures and multicellular algal cultures under high and low light levels.
Lundgren, Veronica. "Grazer-induced de	*Phaeocystis globosa*	The study did not directly assess growth rates of single-celled algae and colonial forms under the different levels of resource availability.
Lurling, M., and W. Beekman. "Palmello	*Chlamydomonas reinhardtii*	Live predators were used instead of predator products/supernatant/kairomones, therefore we can't disentangle whether differences in algal growth are due to predator grazing or resource availability.
Lürling, M. F. L. L. W. "Investigation of	*Scenedesmus pectinatus*	Live predators are inside the 'multicellular' treatments. Thus, growth rates are not only affected by resource availability, but also by grazing.
Lürling, M. F. L. L. W. "Effects of a surf	*Scenedesmus SAG 276/3a*	The comparisons between growth rates of unicellular and multicellular cultures under different nutrient conditions are not independent treatments.
Marchant, H. J. "Colony formation and i	*Eudorina elegans*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Monahan, Thomas J. "Effects of organic	*Scenedesmus obtusiusculus*	The study did not directly assess growth rates of single-celled algae and colonial forms under different nutrients.
Pančić, Marina, and Thomas Kiørboe. "P	several phytoplankton & zooklankton	A review. Provides a summary of published data that I already include in the meta-analysis.
Peperzak, L., et al. "Phytoplankton sinki	*Phaeocystis*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Peperzak, Louis. "Daily irradiance gover	*Phaeocystis*	The study did not assess growth rates of both single-celled algae and colonial forms under high and low light conditions.
Ploug, Helle, Willem Stolte, and Bo Bark	Phaeocystis	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Riegman, Roel, Anna AM Noordeloos, a	several marine phytoplankton	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Shi, X. L., et al. "Survival of Microcystis	*Microcystis aeruginosa* & *Scenedesmus obliquus*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
SIVER, PETER A., and STANLEY J. FF	*Scenedesmus*	The study did not assess growth rates of both colonial forms and single-celled algae under different resources.
Thornton, DCO; Thake, B 1998; Effect o	*Skeletonema costatum*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Tilman, David, Susan Soltau Kilham, and	*Asterionella formosa*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Trainor, Francis R. "Scenedesmus morph	*Scenedesmus*	The study did not assess growth rates of single-celled algae and colonial forms under different resources.
Van Donk, Ellen. "Defenses in phytoplan	*Scenedesmus*	A review. Provides a summary of published data that I already include in the meta-analysis.
Veldhuis, M. J. W., and W. Admiraal. "In	*Phaeocystis pouchetii*	No values of standard deviation/standard error/P-value/F-value reported in the comparison of growth rates of single-celled algae and colonial forms under different levels of phosphate.
Wang, Xiaodong, Yan Wang, and Walker	*Phaeocystis globosa*	There were many different nutrient media and fluorescent light conditions used across papers in the literature. Therefore, if a paper such as this only reported growth rates of single-celled and multicellular cultures in a specific nutrient media (e.g. high nitrate) but not low/medium nitrate, we were not able to classify the high nitrate as "0" or "1" or place it in the continuum between 0-1. Therefore, in the cost meta-analysis, we would only include a study that compares growth rates of single-celled and multicellular cultures in different resource availability (high or low light availability/nutrients) with all other factors in the experimental comparison being equal.
Yang, Zhou; Kong, Fanxiang; Shi, Xiaoli	*Scenedesmus obliquus*	There were many different nutrient media and fluorescent light conditions used across papers in the literature. Therefore, if a paper such as this only reported growth rates of single-celled and multicellular cultures in a specific nutrient media and fluorescent light condition, we were not able to classify this resource availability as "0" or "1" or place it in the continuum between 0-1. Therefore, in the cost meta-analysis, we would only include a study that compares growth rates of single-celled and multicellular cultures in different resource availability (high or low light availability/nutrients) with all other factors in the experimental comparison being equal.
Yokota, Kiyoko, and Robert W. Sterner. '	*Desmodesmus subspicatus*	There were many different nutrient media and fluorescent light conditions used across papers in the literature. Therefore, if a paper such as this only reported growth rates of single-celled and multicellular cultures in a specific nutrient media and fluorescent light condition, we were not able to classify this resource availability as "0" or "1" or place it in the continuum between 0-1. Therefore, in the cost meta-analysis, we would only include a study that compares growth rates of single-celled and multicellular cultures in different resource availability (high or low light availability/nutrients) with all other factors in the experimental comparison being equal.
Yokota, Kiyoko. *Kairomone-induced col*	various phytoplankton	There were many different nutrient media and fluorescent light conditions used across papers in the literature. Therefore, if a paper such as this only reported growth rates of single-celled and multicellular cultures in a specific nutrient media and fluorescent light condition, we were not able to classify this resource availability as "0" or "1" or place it in the continuum between 0-1. Therefore, in the cost meta-analysis, we would only include a study that compares growth rates of single-celled and multicellular cultures in different resource availability (high or low light availability/nutrients) with all other factors in the experimental comparison being equal.

Movie S4.1. (https://www.youtube.com/watch?v=83MAwPoPCCc) This movie shows the flagellate predator *Ochromonas danica* unable to ingest a multicellular group of the algae *Chlorella sorokiniana*.

Chapter 5: Benefit of algal group formation upon ingestion by the predator *Daphnia*: better ability to kill the predator

Delicious 'choco-clumps' I made for my lab-mates on my birthday. The predators (*Daphnia*) came and ate them...Goodbye clumps, what a great adventure it has been - I'll miss you!

But wait, will I see you again at the end of the tunnel?!

Abstract

The first step in the evolution of multicellularity is group formation. This leads to the question of why single-celled organisms form groups. I have shown in the algae *Chlorella sorokiniana*, that the formation of groups reduces ingestion by a small predator, the flagellate *Ochromonas*, but does not reduce ingestion by a larger predator, the crustacean *Daphnia magna*. So why do individuals of *C. sorokiniana* form groups in the presence of *D. magna*? We found that when exposed to *Daphnia* products: (1) *C. sorokiniana* cells form sticky multicellular groups that the mobile predator *D. magna* ingests; (2) some algae can survive inside the gut of the predator and pass alive through the digestive track; and (3) the ingested multicellular groups are better able than unicells to kill these predators. This advantage of algal group formation in terms of better ability to kill their predator may provide an additional explanation to the evolution of multicellular groups.

Introduction

The evolution of multicellularity throughout the tree of life began by single cells forming a group (Bourke 2011; West et al. 2015). Why would single cells invest energy, such as the production of adhesive extracellular material and/or have less access to nutrients/sunlight, in forming a cooperative group (Reynolds 1984; Lancelot and Mathot 1985; Kirk 1994; Trainor 1998; Ploug et al. 1999; Lürling 1999; Tollrian and Dodson 1999; Lürling and Van Donk 2000; Chapter 4)? This step of single cells coming together can be beneficial when food is scarce, such as in the case of stalk formation in slime moulds or wolf-pack feeding in myxobacteria (Dworkin and Bonner 1972; Velicer and Yuen-tsu 2003; Berleman and Kirby 2009; Smith et al. 2014), and even when predators are present (Hessen and Van Donk 1993; Boraas et al. 1998; Grosberg and Strathmann 2007; Claessen et al. 2014; Kapsetaki et al. 2016;

Herron et al. 2018; Chapter 4). Forming a group in algae is most often known to provide a benefit in terms of protection from ingestion by small predators, such as *Ochromonas* (Boraas et al. 1998; Pančić and Kiørboe 2018; Chapter 4).

However, *C. sorokiniana* forms groups in response to the large predator *Daphnia* even though there appears to be no survival benefit, i.e. multicellular and single-celled algae of *C. sorokiniana* are ingested at roughly equal amounts by the large predator *D. magna* (Chapter 4). So why does *C. sorokiniana* form groups in response to *Daphnia*? One explanation may be that groups are better than single cells at surviving through the predator's gut, as is known for several prey species across the tree of life (Table 5.1). Specifically, researchers have suggested that, once ingested, multicellular algal groups of *Chlorella vulgaris* and *Sphaerocystis shroeteri* can survive digestion through the *Daphnia*'s gut (Ryther 1954; Porter 1976; Lampert 1987; Kampe et al. 2007). Such algal-predator relationships could be mutualistic or parasitic. In the former case, the resistant algal groups may be better than single cells at trading photosynthates in return for phosphorus, other nutrients from algal remains or from *Daphnia* metabolites inside the gut, and gut passage (Porter 1976; Epp and Lewis 1981). In the latter, multicellular algae do not provide photosynthates in return, and are better than unicells at using *Daphnia's* luxurious localised source of nutrients in the gut (Porter 1976; Gladyshev et al. 2000), and/or surviving digestion in the gut, due to their group size or hardness, such as the formation of a protective gelatinous extracellular sheath (DeMott 1995). According to Van Donk et al. (1999), "large gelatinous colonial chlorophytes may be ingested, but are hardly digested by zooplankters like *Daphnia* (Porter 1976), resulting in depressed zooplankton growth rates (Vanni and Lampert 1992; Stutzman 1995)".

Table 5.1. Examples of prey surviving passed the predator's digestive track.

Prey	Predators	References
Pelagic larvae of lamellibranchs	Polychaete (*Chaetopterus variopedatus*)	(MacGinitie 1939)
Nematode worms (*Caenorhabditis elegans*)	Slugs	(Petersen et al. 2015)
Plant seeds & wasps (*Megastigmus aculeatus nigroflavus*) inside seeds	Mockingbird (*Mimus polyglottos*)	(Nalepa and Piper 1994)
Bromeliad ostracods (*Elpidium*)	Tadpoles (*Scinax perpusillus*), mouse	(Lopez et al. 2002)
Shells (*Mytilus*)	Herring gull (*Larus argentatus*), Oystercatcher bird (*Haematopus ostralegus*)	(Cadée 1989; Brown and Kotler 2007)
Snails (*Tornatellides boeningi*)	Japanese white-eye (*Zosterops japonicus*) and the brown-eared bulbul bird (*Hypsipetes amaurotis*)	(Wada et al. 2012)
Oysters (*Crassostrea gigas*)	Oystercatchers (*Haematopus ostralegus*)	(Cadée 2008)
Gastropod mollusks	Fish (*Asemichthys taylori*)	(Norton 1988)
Cycolopoid eggs	Bluegill sunfish	(Vinyard 1979)

(*Cypriodopsis vidua*)		
Copepod eggs (*Eurytemora*, *Euterpina*)	Turbot fish (*Scophthalnius maximus*)	(Conway et al. 1994)
Calanoid eggs (*Eurytemora*)	Baltic herring fish (*Clupea harengus membras*)	(Flinkman et al. 1994)
Eggs of copepods, cladocerans, and rotifers	Larvae of catfish (*Heterobranchus longifilis*)	(Saint-Jean and Pagano 1995)
Crustacean eggs (*Bythotrephes cederstroemi*)	Fish	(Jarnagin et al. 2000)
Copepod eggs (*Eudiaptomus*, *Cyclops*, *Macrocyclops*)	Fish	(Bartholmeé et al. 2005)
Mussels (*Dreissena*)	Round goby (*Neogobius melanostomus*)	(Mack and Andraso 2015)
Adult clams and gastropods	Sea star (*Astropecten irregularis*)	(Christensen 1970)
Opisthobranchs	Sea slugs (*Navanax inermis*)	(Paine 2011)
Mussels (*Mytilus edulis*)	Sea anemones (*Actinia equina*)	(Davenport et al. 2011)

Diatoms	Larval caddisfly (*Neophylax autumnus*)	(Peterson 1987)
Diatoms	Crabs (*Aegla uruguayana*)	(Devercelli and Williner 2006)
Diatoms	Gastropods	(Nicotri 1977)
Diatoms	Gastropods (*Amphibola crenata*)	(McClatchie et al. 1982)
Green algae (*Botryococcus*)	Crustaceans (*Gammarus lacustris*)	(Gladyshev et al. 2000)
Gelatinous green algae	Cladoceran (*Daphnia*)	(Porter 1975)
Algae	Tadpoles (*Limnodynastes tasmaniensis*)	(Peterson and Boulton 1999)
Bacteria and epiphytic algae	Snails (*Lymnaea*, *Planorbis*)	(Underwood and Thomas 1990)
Bacteria, algae, and small zooplankton	Copepods	(Turner and Ferrante 1979)
Bacteria (*Chromobacterium violaceum*)	Flagellates (*Ochromonas*)	(Matz et al. 2004)

We examine whether multicellular group formation in the Chlorophyte algae *C. sorokiniana* helps algal cells survive passage through the gut of their predator *D.*

magna, and/or helps algal cells to kill this predator. Specifically, we test: (1) whether multicellular algal groups versus single cells have an extracellular gelatinous sheath; (2) whether algae can remain alive inside the *Daphnia's* gut; (3) whether algae can survive passage through a *Daphnia*'s gut; (4) whether multicellular versus unicellular algae have higher survival rates during passage through the *Daphnia* gut; and (5) whether ingestion of multicellular or unicellular algae affects *Daphnia* survival rates.

Methods

Strains

We used the algae *Chlorella sorokiniana* (non-axenic from the Culture Collection of Algae and Protozoa; strain number 211/8K) as prey, grown in Bolds Basal media (Sigma) at 20^{o}C and a light:dark cycle of 16:8 hours fluorescent illumination. In order 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 samples 1:300 after 24 hours in Bolds Basal media (Kapsetaki et al. 2016; Kapsetaki et al. 2017; Chapter 4). Before using the algae in experiments, we maintained cultures in 1-L Erlenmeyer flasks shaking at 220 rpm, at 20^{o}C and a light:dark cycle of 16:8 hours fluorescent illumination.

We used *Daphnia magna* (Sciento, UK) as predators, which we fed 5 mL *Scenedesmus obliquus* (10^{6} cells mL^{-1}) every 4-5 days. We maintained the *D. magna* in 500-mL jars and at 20^{o}C and a light:dark cycle of 16:8 hours fluorescent illumination.

Observations of algae on the sides of tubes and Scanning Electron Microscopy imaging

We examined whether *Daphnia* products induce the algae *C. sorokiniana* to form: (1) multicellular groups; (2) groups adhesive to plastic; (3) groups surrounded by an extracellular sheath; and (4) whether live *Daphnia* can remove and ingest the groups adhered to the plastic. We transferred 4.04 mL of *C. sorokiniana* (10^6 cells mL^{-1}) to either 0.96 mL of filtered Bolds media in Falcon tubes (BD Biosciences), or 0.96 mL of filtered liquid from the culture of *D. magna* (final concentration of 3 individuals per mL) in Falcon tubes. We used 25 independent replicates per treatment, randomized tubes on tube racks and incubated tubes at 20^{o}C at a light:dark cycle of 16:8 hours fluorescent illumination, keeping the tube caps loose to allow for oxygenation.

First, to test whether *Daphnia* products induce algal group formation, after adding the predator treatments, we collected samples at 0 and 96 hours by tilting the tubes five times 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) at x20 magnification. We measured the total number of algal cells and the proportion of cells in groups; as group we define ≥3 cells in close proximity. We counted paired cells as single cells in all experiments.

Second, to test whether algae adhere to the sides of tubes, we took photos of the bottom half of the tilted tubes using a digital camera (Canon PowerShot A2600) at 0 and 96 hours. We took separate photos including one tube per treatment. In each of these photos we selected a rectangular area from the cone-shaped region of each tube, that had no bright reflection from the light and measured its greenness index [greenness index = mean green intensity/(mean red intensity + mean blue intensity + mean green intensity)] using ImageJ. At the final 96-hour time point we also performed the following experiments.

Third, we examined the sides of tubes at high-resolution microscopy for the presence of any adhesive algal cells and an extracellular sheath surrounding groups and/or single cells. We discarded the liquid culture from three Falcon tubes from the treatments of algae with filtered Bolds media, and algae with filtered *Daphnia* products. We carefully removed a small cuboid piece of plastic (~ 4 mm) from the bottom of each tube using a pre-heated scalpel, and sent these samples for imaging under a Scanning Electron Microscope (SEM). The tube samples were connected to a metal stub using Silver DAG and coated with 5 nm platina. This final SEM sample-preparation step and SEM images were obtained by Koen Evers (Department of Materials, University of Oxford). In these microscopy images we measured the total number of algal cells and the proportion of cells in groups.

Fourth, we tested whether *Daphnia* can remove and ingest the observed green material from the sides of tubes. We removed the liquid culture from 12 tubes of *C. sorokiniana* with *Daphnia* products, added 4 mL filtered *Daphnia* media with 15 *Daphnia* in six tubes, and 5 mL filtered *Daphnia* media in the other six tubes. The filter used in all experiments had a pore diameter of 0.22 μm. We left the 12 tubes for four hours at 20^{o}C and light:dark cycle of 16:8 hours fluorescent illumination. We tilted the tubes fives times and obtained images of the bottom half of the tubes prior- and post-addition of the *Daphnia* using a digital camera (Canon PowerShot A2600) and measured the tubes' greenness index, and obtained images of the bottom section of three tubes per treatment after four hours using SEM. We also obtained images of each *Daphnia*'s gut prior and 4 hours after adding the *Daphnia* to the tubes using a digital camera (Canon PowerShot A2600), and measured their greenness index in the beginning of the experiment and after 4 hours by averaging the 15 gut greenness index values per tube to a single data point (n = 6) at each time point.

Testing whether algae can survive inside the *Daphnia*'s gut

In order to examine whether algae can survive inside the gut of the *Daphnia,* we first collected 35 *Daphnia* and left them in 2 mL Bolds media in the dark overnight at room temperature. We then fed the surviving *Daphnia* 1 mL *C. sorokiniana* (10^6 cells mL^{-1}) for 10 minutes, washed each *Daphnia* 3 times in 2 mL dH_2O, and left them for 3 hours in dH_2O. We transferred each *Daphnia* on a separate 1% agar-Bolds Petri dish for 48 hours at 20°C and 16:8 light dark cycle until the *Daphnia* died and their gut contents were immobile. We then obtained images once every day for three days under an inverted microscope (magnification: X10) to measure the number of algae in a particular location in their gut over time. The majority of the *Daphnia* left on the Petri dish either had body sections, such as the gut, broken/dislocated during the process of dying/drying up on the Petri dish, or their gut contents were too dense to distinguish individual algal cells. Only in 3 out of 35 *Daphnia,* cells of *C. sorokiniana* were clearly distinguishable and measurable inside the gut. Measurements of the number of algal cells were taken in a section of the lower region of the gut, which was consistent across individuals and across time, because the anterior and middle area of the gut were most often surrounded by other dense body segments, thus not allowing clear observation of the gut's contents.

Testing whether algae can survive passed the *Daphnia*'s gut

We examined whether: (i) unicellular algae and multicellular algae can survive passing through the gut of a *Daphnia*; (ii) the survival of multicellular groups is higher than that of single cells when passing through the gut of a *Daphnia*; and (iii) eating multicellular groups leads to higher death rates in *Daphnia* than eating single cells. We followed a similar protocol to Porter (1976), who studied the survival of *Sphaerocystis* algae passed the gut of *Daphnia*, but not *Daphnia* death rates. We prepared unicellular and multicellular algal cultures by adding filtered Bolds media or *Daphnia* products to a culture of *C. sorokiniana* for 96 hours, respectively. After the 81-hour time point, we starved 293 *Daphnia* for 15 hours in 2 mL dH_2O in the dark (24-well plates covered in aluminum foil) to remove *S. obliquus*, and washed each *Daphnia* twice in 50 mL dH_2O. We fed each of 50 *Daphnia* 1 mL unicellular *C. sorokiniana* (10^6 cells mL^{-1}), each of the other 50 *Daphnia* with 1 mL multicellular culture of *C. sorokiniana* (10^6 cells mL^{-1}), and each of the remaining 193 *Daphnia* were fed no algae for 30 min. We washed each *Daphnia* twice with 50 mL dH_2O, and kept each *Daphnia* in the dark in 2 mL filtered *Daphnia* media until the *Daphnia* moulted. We checked for moulting every seven hours. During these starvation, washing, and moulting steps, 185 *Daphnia* died in total. When a *Daphnia* had moulted, we collected the live *Daphnia* and not its exoskeleton, washed the *Daphnia* twice in 50 mL dH_2O, and left the *Daphnia* for 2 days in 2 mL filtered *Daphnia* media. After 2 days we noted the number of dead and alive *Daphnia*. We then collected 1 mL of the medium in Eppendorf tubes, without collecting the dead/alive *Daphnia* or any exoskeleton if present, and left the medium for seven days in 16:8 hour light:dark conditions at 20^oC to allow algal growth, and at the final time point observed each Eppendorf tube to check for the presence of any *C. sorokiniana*.

Statistical analysis

For all analyses we used R version 3.2.3 (Crawley 2012; R Core Team. 2015). We analysed the difference in the number of algae on the sides of tubes, and the number of algae in the liquid culture (filtered Bolds media and *Daphnia* supernatant treatments, or *Daphnia* supernatant and *Daphnia* supernatant with live *Daphnia*) using a generalized linear model ('glm' package) with Gaussian errors to account for data overdispersion. For estimating the difference in the greenness index, the proportion of algae on the sides of tubes, and the proportion of algae in groups in the liquid culture between the treatments, we used the same model but specified family as quasibinomial, to account for overdispersion of the data.

When data were repeated measurements from the same individuals or cultures (experiments testing whether "Algae can be alive inside the *Daphnia*'s gut" and "*Daphnia*'s gut greenness index") we compared algal numbers over time using a generalized mixed-effects model with Penalized Quasi-Likelihood ('glmmPQL' package) and Gaussian errors; for comparing the gut greenness index over time we used the same generalized mixed-effects model (glmmPQL), but with quasibinomial errors. We treated time as a fixed effect, and the repeated measurements as random effects (1 | Subject).

We performed Pearson's chi-square test to compare death rates of *Daphnia* fed unicellular, multicellular, or no *C. sorokiniana*. For analysing differences in survival rates of unicellular versus multicellular algae passed the predator's gut, instead of Pearson's chi-square test, we used Fisher's exact test due to small sample sizes.

Results

Predator products induce algal group formation and stickiness of algal groups to plastic

As in our previous experiments, we found that algal cultures exposed to *Daphnia* supernatant form multicellular groups in liquid culture (Fig. 5.1F; generalized linear model (glm) by 96 hours, $F_{1,48}$ = 263.42, $P < 0.0001$; non significant difference between treatments in the proportion of cells in groups in the beginning of the experiment, glm at 0 hours, $F_{1,48}$ = 0.03, P = 0.86). Cultures exposed to *Daphnia* supernatant contained similar amounts of total algae in the culture compared to cultures exposed to filtered media (Fig. 5.1E, glm, $F_{1,48}$ = 2.01, P = 0.16; non significant difference between treatments in the beginning of the experiment, glm at 0 hours, $F_{1,48}$ = 0.11, P = 0.73).

Also, algae with *Daphnia* supernatant displayed greater 'stickiness' to the plastic tubes, as shown by the higher greenness index (Fig. 5.1A; glm at 96 hours, $F_{1,48}$ = 41.84, $P < 0.0001$; non significant difference in greenness in the beginning of the experiment, glm at 0 hours, $F_{1,48}$ = 2.38, P = 0.13). Imaging of tube sections from the two treatments under a Scanning Electron Microscope clearly revealed the presence of an extracellular sheath surrounding the multicellular groups, though this sheath was not as clearly visible around unicells (Fig. 5.1B). Quantification of algal cell numbers and proportion of cells in groups from these images revealed that more cells are stuck on the sides of tubes containing cultures exposed to *Daphnia* supernatant than cultures exposed to just filtered Bolds media (Fig. 5.1C, glm, $F_{1,4}$ = 523.8, $P < 0.0001$), and these algae on the sides of the tubes are predominantly multicellular (Fig. 5.1D, glm, $F_{1,4}$ = 130.61, $P < 0.001$).

Daphnia can remove and ingest the sticky green material from the sides of tubes

The observed green material, number of algae, and proportion of cells in groups on the sides of tubes, was reduced in the presence of live *Daphnia* (Fig. 5.2A; glm, $F_{1,10} = 43.16$, $P < 0.0001$; Fig. 5.2B; Fig. 5.2C, glm, $F_{1,4} = 521.43$, $P < 0.0001$; Fig. 5.2D, glm, $F_{1,4} = 130.61$, $P < 0.001$), and the greenness index of the *Daphnias'* gut was increased, indicating ingestion of the algae (Fig. 5.2E, glmmPQL, $n_{total} = 6$, $P = 0.0002$).

Algae can be alive inside the *Daphnia*'s gut

Cells of *C. sorokiniana* were visible inside the gut of dead *Daphnia* (Fig. 5.3). Over three days, the number of algal cells increased significantly, implying that algal cells can survive in the gut (Fig. 5.3, glmmPQL 48 versus 96 hours, $n_{total} = 3$, $P = 0.002$).

Algal survival through *Daphnia*'s gut and higher *Daphnia* death rates when fed multicellular algae

After feeding the *Daphnia* unicellular or multicellular *C. sorokiniana* for 30 minutes, the *Daphnias*' guts were green, indicating ingestion (n = 100). When *Daphnia* were fed *C. sorokiniana*, and the *Daphnia* were washed several times, left to moult and survived moulting, the majority of tubes did not contain algae (Table 5.2; 22 tubes out of 28, 12 out of 13, 9 out of 10, and 23 out of 26), indicating digestion of the algae by the *Daphnia*. However, we saw algae in 21.4% of the tubes when *Daphnia* were fed unicellular *C. sorokiniana* (Table 5.2; 6 out of 28 tubes), and algae in 7.6% of the tubes (1 out of 13 tubes) when *Daphnia* were fed multicellular *C. sorokiniana,* indicating algal survival passed the gut. These survival rates of unicellular versus multicellular algae passing through the *Daphnia*'s gut did not differ significantly (Table 5.2; Fisher's exact test: $P = 0.09$). Still, the *Daphnia* died at higher rates when they were fed multicellular rather than unicellular *C. sorokiniana*; with the highest death rates appearing when the *Daphnia* were not fed any algae (Table 5.2; chi-square test: $\chi^2 = 39.9$, $df = 2$, $P < 0.0001$).

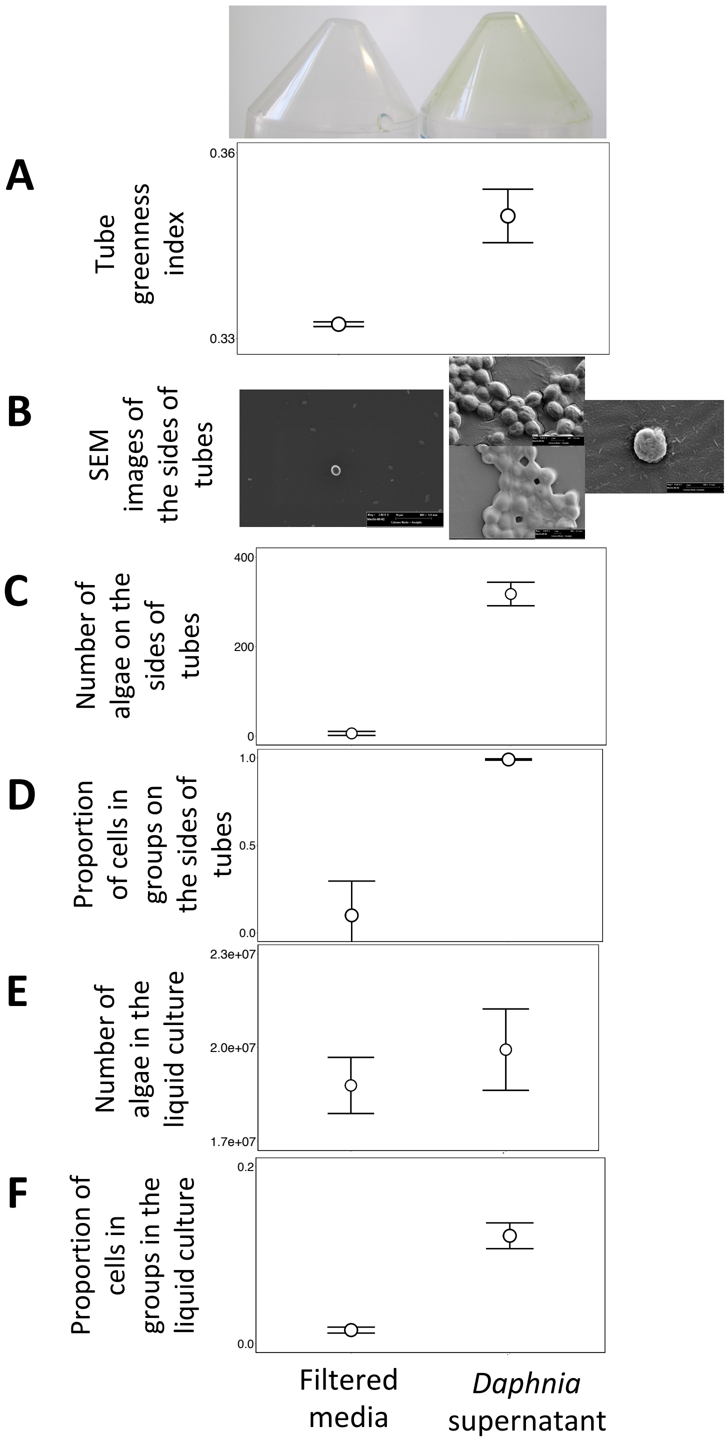


Figure 5.1. Predator products induce multicellular group formation in *C. sorokiniana* and stickiness of algal groups to plastic. Plots show results 96 hours

after exposure of algal cultures to either filtered media or *Daphnia* supernatant. The greenness index of tubes (25 independent replicates per treatment (irpt)) (**A**), the number of algae (n_{irpt} = 3) (**C**) and proportion of cells in groups on the sides of tubes (n_{irpt} = 3) (**D**), the number of algae (**E**) (n_{irpt} = 25) and proportion of cells in groups in the liquid culture (n_{irpt} = 25) (**F**) is higher in algal cultures exposed to *Daphnia* supernatant than filtered media. (**B**) Representative Scanning Electron Microscope images of tube sections from the two treatments. Error bars show 95% confidence intervals (CI).

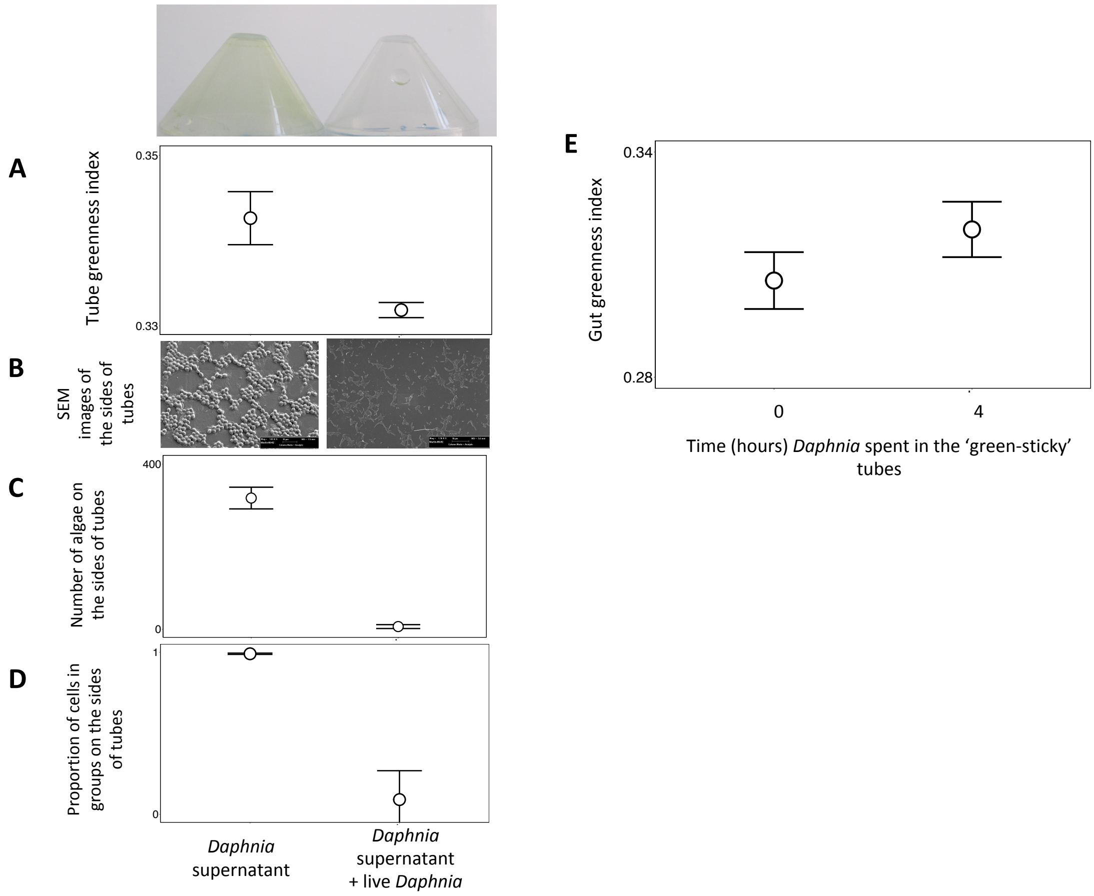


Figure 5.2. *Daphnia* can remove and ingest the sticky green algae from the sides of tubes. Plots **A-D** show results of 96-hour-old algal cultures, four hours after exposure to either *Daphnia* supernatant, or *Daphnia* supernatant with live *Daphnia*. The tube greenness index (n_{irpr} = 6) (**A**), the number of algae (n_{irpr} = 3) (**C**), and the

proportion of cells in groups on the sides of tubes (n_{irpr} = 3) (**D**), decreases in the presence of live *Daphnia*. The greenness index of the *Daphnia*'s guts increases four hours after *Daphnia* are placed inside the 'green-sticky' tubes, indicating ingestion of the green algae (independent replicates in total (n_{total}) = 6) (**E**). Error bars show 95% CI.

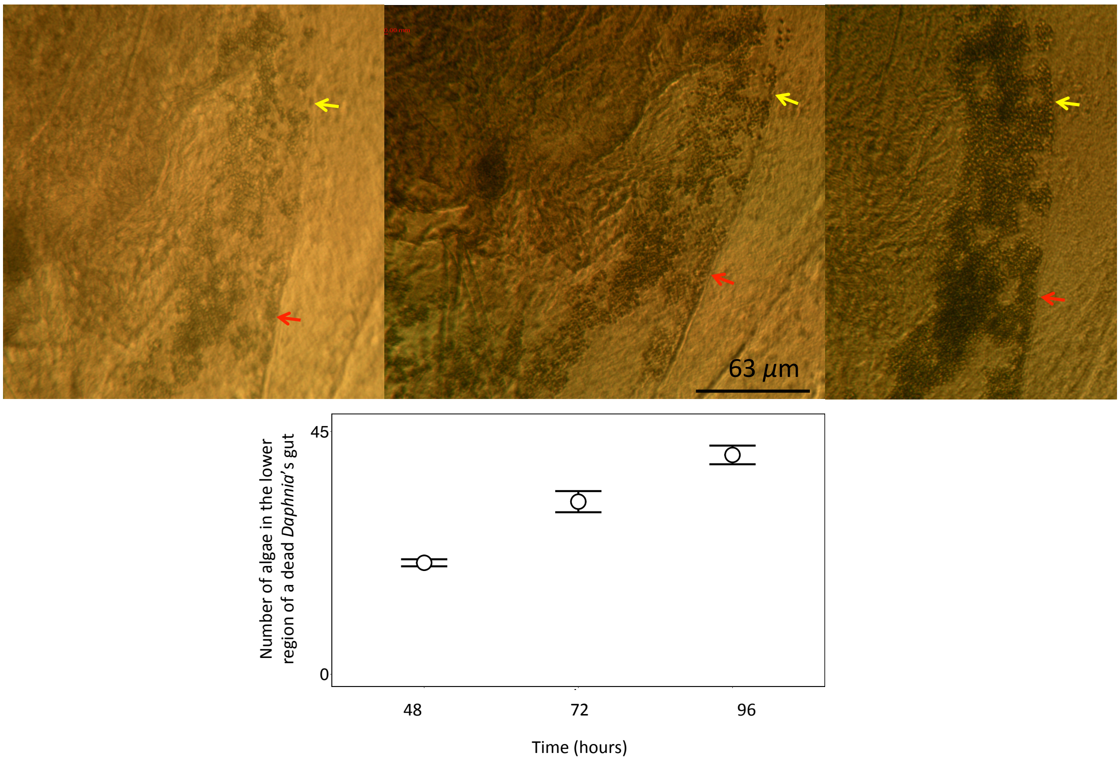


Figure 5.3. Algal growth inside the gut of dead *Daphnia*. The number of algae in the lower region of the gut increases over three days (n_{total} = 3). Error bars show 95% CI. Representative images are shown at each time point. Arrows indicate some areas of algal growth over time.

Daphnia were fed:	Number of wells in which *Daphnia* was alive			Number of wells in which *Daphnia* was dead			% Wells in which *Daphnia* was dead
	Number of tubes with algae	Number of tubes w/o algae	% Tubes with algae	Number of tubes with algae	Number of tubes w/o algae	% Tubes with algae	
Unicellular *C. sorokiniana*	6	22	**21.4**	1	9	**10.0**	**26.3**
Multicellular *C. sorokiniana*	1	12	**7.6**	3	23	**11.5**	**66.6**
No algae	0	0	**NA**	0	31	**0.0**	**100.0**

Table 5.2. Effect of eating unicellular, multicellular, or no *C. sorokiniana* on the death rates of *D. magna* (bold black), and percentage of tubes with algae indicating algal survival through the gut of live (bold dark green) and dead *Daphnia* (bold light green).

Discussion

C. sorokiniana forms multicellular groups in response to predator products from *Daphnia* (Fig. 5.1F; Kapsetaki et al. 2016; Kapsetaki et al. 2017; Chapter 4). We also found that: (1) *Daphnia* products induce stickiness of algal groups to plastic (Fig. 5.1); (2) *Daphnia* can remove and ingest these sticky algal groups from the sides of tubes (Fig. 5.2); (3) algae can be alive inside the *Daphnia*'s gut (Fig. 5.3); (4) algae can survive passage through the *Daphnia*'s gut (Table 5.2); and (5) algal groups are more likely than unicells to kill their predator *D. magna* (Table 5.2). These findings in the Chlorophyte algae *C. sorokiniana* follow almost exactly Van Donk et al. (1999) description that "large gelatinous colonial chlorophytes may be ingested, but are hardly digested by zooplankters like *Daphnia* (Porter 1976), resulting in depressed zooplankton growth rates (Vanni and Lampert 1992; Stutzman 1995)".

Survival inside and passed the predator's gut

Our observation of algal growth inside the gut of dead *Daphnia* (Fig. 5.3) might be an indication that algae can survive in the gut, or an indication that they had not been yet/fully digested when the *Daphnia* died. *Daphnia* is a small crustacean that is mostly transparent, so the ingested algae (Fig. 5.2 & 5.3) could potentially still be exposed to sunlight and thus be able to grow inside the gut if they have not been digested by the *Daphnia*. In fact, colonial algae of *Sphaerocystis* can gain nutrients inside the *Daphnia*'s gut, which stimulates algal carbon fixation and cell division (Barlow and Bishop 1965; Porter 1976).

Approximately 10-20% of algae were able to survive passage through the gut of *Daphnia* (Table 5.2). Similar survival rates have been observed in several other predator-prey species. For instance, 14.3% and 16.4% of snails survive passed the gut of Japanese white-eyed birds and brown-eared bulbuls (Wada et al. 2012), 53 to 75% of Ostracoda, 46 to 92% of *Hydrobia spp*., 94% of *B. cederstroemi* eggs, and 26% of the ostracods *Cypridopsis* can survive passed the gut of fish (Vinyard 1979; Aarnio and Bonsdorff 1997; Jarnagin et al. 2000; Jarnagin et al. 2004). Also, more than 90% of the green colonial algae *Sphaerocystis schroeteri* ingested by *D. magna* can survive undamaged passed the predator's gut (Porter 1976), and when these algae are abundant they can be ingested multiple times, one to four, per day by the *Daphnia* (Haney 1973).

Survival passed the predator's gut as a means of prey dispersal

C. sorokiniana are immobile algae, whereas their predator *D. magna* is mobile. Therefore, *C. sorokiniana* may disperse to further locations whilst in the gut of *Daphnia*. Throughout the tree of life, dispersal of prey by predators has been seen in ostracods, mudsnails, mollusks, snails, and eggs of crustaceans dispersed by fish and birds (Norton 1988; Aarnio and Bonsdorff 1997; Jarnagin et al. 2004; Brown and Kotler 2007; van Leeuwen et al. 2012; Van Leeuwen et al. 2012; van Leeuwen and van der Velde 2012; Wada et al. 2012). Also, *Daphnia* eggs can be dispersed via fish-eating birds and mammals feeding on invertebrate predators of *Daphnia* (Mellors 1975). Seeds and eggs of the aquatic beetle *Macroplea mutica* can be dispersed via ducks across 75 km (Figuerola and Green 2002; Laux and Koelsch 2014), and seeds of the fig *Ficus glabrata* can travel unaffected passed the gut of fish across a 6 km-river (Horn 1997).

Multicellular groups are better able than single cells to kill their predator

Once dispersed by the predator *Daphnia*, algal groups may be better than unicells at killing their predator as a means of escape. Prey often kill their predator, either by producing toxins or by causing predator starvation by being indigestible. When bacteria are ingested by the flagellate predator *Ochromonas*, they can escape by killing the predator with toxins (Matz et al. 2004). In our experiments, we found higher death rates when *D. magna* had ingested multicellular algal groups of *C. sorokiniana* rather than single cells. We know that *Daphnia* decrease algal density in both unicellular and multicellular cultures of *C. sorokiniana* indicating almost equal ingestion of unicells and multicellular groups (Chapter 4). Thus, the difference in death rates between *Daphnia* exposed to unicells or multicellular cultures (Table 5.2) cannot be explained due to differences in *Daphnia* ingestion rates. There may be differences in the digestibility of multicellular groups versus single cells. We observed stickiness of algal groups to plastic, similar to previous findings of *C. sorokiniana* biofilms having elevated attachment to polystyrene foam and polycarbonate disks (Fig. 5.1; Johnson and Wen 2010; Blanken et al. 2014), but have not yet identified the exact nature of the 'sticky' substance. The 'sticky' substance may be an extracellular sheath produced by the algae *C. sorokiniana* in multicellular groups and surrounding them, as has been previously seen surrounding colonies of

Chlorella vulgaris (Boraas et al. 1998), but not in single cells (Fig. 5.1B, 5.1C, 5.1D). Apparently, the extracellular gelatinous sheet surrounding algal colonies can protect algae from digestion by the predators *D. magna* (Porter 1975; Porter 1976). For instance, colonies of the green algae *Botryococcus* with a thick mucilaginous sheath survive through the gut of the small crustacean *Gammarus* (Gladyshev et al. 2000), and snails probably resist digestion in bird's guts due to their shell and mucus protecting them from the acidic gut environment (Wada et al. 2012). Nongelatinous single-celled algae such as cryptomonads, diatoms, and nannoplankton, and fungal zoospores without thick cell walls or sheaths are easily digested by *Daphnia* and other zooplankton (Porter 1975; Porter 1976; Beakes et al. 1988). We did not observe differences in the digestibility of unicells and multicellular groups as measured in terms of algal survival passed the *Daphnia's* gut (Table 5.2). However, we observed higher death rates in *Daphnia* fed multicellular *C. sorokiniana*, which could indirectly indicate differences in digestibility. Multicellular groups with their gelatinous sheath may be less digestible than unicells or they may be causing blockage in certain regions of the gut (e.g. Movie S5.1), leading to *Daphnia* malnutrition, starvation and eventually death (Van Donk et al. 1999). In summary, once the multicellular algal groups inside the gut have used the predator to disperse and/or acquire nutrients, they may kill their predator *Daphnia* as a mechanism of escape.

Future studies testing whether algal ingestion by predators actually favours algal dispersal, whether groups are better than single in gaining nutrients from the gut, and whether the algae provide anything in return to the *Daphnia*, would help us better understand these algal-predator interactions and the benefit of algal multicellular group formation upon exposure to *Daphnia*.

Acknowledgements

We would like to thank: Qi Qin for comments on the manuscript; Koen Evers for obtaining images under the Scanning Electron Microscope; Dieter Ebert for valuable advice on the protocol testing whether algae can pass alive through the *Daphnia*'s gut; and Nick Harberd and Lindsay Turnbull for providing temporary lab space. This project was funded by the European Research Council (to Stuart West), the Alexander S. Onassis Public Benefit Foundation and the A.G. Leventis Foundation (to Stefania Kapsetaki).

Supplementary Material

Movie S5.1. (https://www.youtube.com/watch?v=F4FXKaEaYCU)

Time-lapse video showing temporal blockage of a clump in the middle region of the oesophagus of *Daphnia magna* that was fed multicellular *Chlorella sorokiniana*.

Chapter 6: General Discussion

Overview

Each previous chapter contains an extensive discussion relevant to that particular study. The aim of this chapter is to summarise the main results in the thesis, discuss them in a more general context, and highlight new directions for future research.

Summary of main results

We found that the algae *C. sorokiniana*, *C. vulgaris,* and *S. obliquus* form multicellular groups in response to the predators *Ochromonas*, *T. thermophila*, and *D. magna* (Chapter 2). In three predator-prey combinations, this group formation could be induced just by predator products (Chapter 2), and these groups formed via aggregation and by remaining stuck to the parent cell after division (Chapter 3). Different algal species could also come together and form multicellular groups (Chapter 3). In chapter 4 we illustrated that this group formation is beneficial when algae are exposed to the small predator *Ochromonas*, but not the larger predator *Daphnia*, and found fitness costs of group formation under limited resources. Finally, in chapter 5 we found that the algae *C. sorokiniana* can survive through the gut of *D. magna*, and group formation may provide a benefit in terms of better ability to kill the *Daphnia*.

Clarification: What is a predator?

Ochromonas, *Tetrahymena*, and *Daphnia*, caused a significant decrease in algal density (Chapters 2 & 4). Still, some algae of *C. sorokiniana* could survive passed the *Daphnia*'s gut (Chapter 5). Therefore, does *Daphnia* fit the definition of a predator of *C. sorokiniana*? According to several dictionaries, a predator is an animal that obtains food by killing and consuming other organisms (Hornby 1974; Dictionary 2006;

Dictionaries 2009; Dictionary 2015). Some of the ingested *C. sorokiniana* were not killed by the *Daphnia*, they survived through its gut, but because the majority of the ingested *C. sorokiniana* were killed by the *Daphnia*, in this thesis we include *Daphnia* in the definition of a predator.

Group formation: additional proximate explanations

Predatory group-inducing cues

An encounter with a predator is usually lethal. In response, organisms have evolved ways of detecting the predator before the close encounter. Humans for instance use their visual, acoustic, and olfactory senses to detect predators (e.g. Fig. 6.1). Similar to olfactory senses, algae have evolved mechanisms detecting chemical messages predictable and reliable of the presence of predators (Dicke and Sabelis 1988; Vet and Dicke 1992; Van Donk et al. 1999; Tollrian and Harvell 1999; Brönmark and Hansson 2000; Wisenden 2000). In chapter 2 I found that *Ochromonas* and *Daphnia* significantly decreased algal density, indicating that they are algal predators, and live predators induced group formation in all nine predator-prey combinations. The algae *C. sorokiniana*, *C. vulgaris*, and *S. obliquus* also formed groups in response to filtered liquid from the predator cultures. We chose this filtering method instead of a specific chemical cue because: (1) a specific predatory group-inducing chemical had not yet been identified for *C. sorokiniana* and *C. vulgaris*; (2) it was far more inexpensive; and (3) we expected it would probably more likely resemble natural circumstances where algae are exposed to a variety of chemicals, and not just one. Still, sophisticated biochemical studies have revealed that in *Scenedesmus*, group formation can be induced by just one predatory molecule; the anionic surfactant 8-methylnonyl sulphate isolated from *Daphnia*, and the similar-structured manmade sodium dodecyl sulphate and FFD-6 (Lürling and Beekman 2002; Lürling 2006). FFD-6 actually induces group formation in *Scenedesmus* without affecting algal growth (Yasumoto et al. 2005; Lürling 2006; Yasumoto et al. 2006; Uchida et al. 2008). Interestingly another chemical, metribuzin, can reverse *Scenedesmus* groups back to single cells by inhibiting algal growth (Lürling 2011). The amphiphilic structure of these info-chemicals provides clues as to how they may function. Amphiphilic means that these molecules possess both hydrophilic and lipophilic groups, where the former allow their dispersal in water, and the latter possibly enable their absorption through the algal cell membrane, respectively (Von Elert and Franck 1999; van Holthoon et al. 2003).

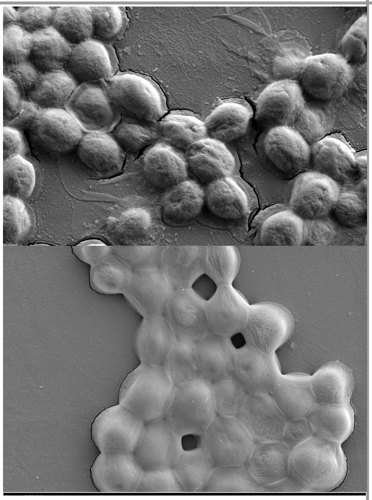
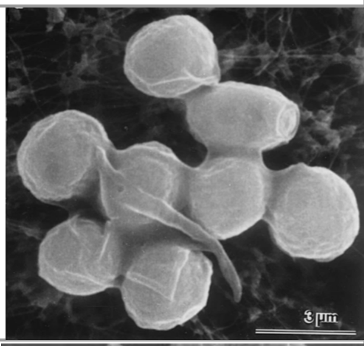
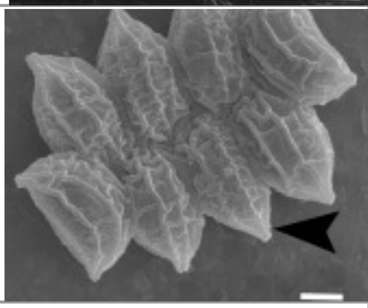
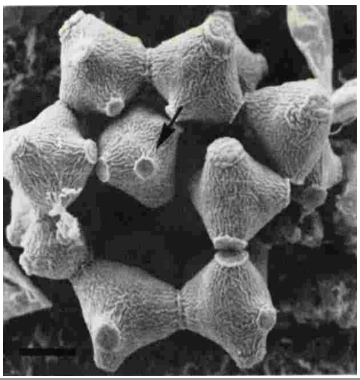
Figure 6.1. *Crocodylus niloticus* (here covered in algae) is responsible for some of the most deadly attacks on humans. Source: Wikipedia Creative Commons Attribution 2.0.

Predator-induced group-adhesive substance

Once the predatory chemical has been detected, algae form groups by aggregating, by remaining attached after division, and even by coming together with different algal species (Chapter 3). Algal groups of *C. sorokiniana* are also able to stick to plastic and are surrounded by an extracellular sheath (Chapter 5). In our study we did not identify the exact nature of this adhesive and/or extracellular substance. However, other studies have shown that there are localised thickened plaques on the cell walls of *Chlorella*, *Scenedesmus*, and *Coelastrum* colonies (Table 6.1), which grow and fuse, facilitating cell aggregation (Atkinson et al. 1972; Marchant 1977; Boraas et al. 1998). Apparently, algae produce extracellular polysaccharides that increase cell stickiness and aggregation (De Philippis and Vincenzini 1998; Van Rijssel et al. 2000; Pajdak-stós et al. 2001; Thornton 2002). Specifically, it is known that in the presence of *Daphnia* water, the parent cell of *Scenedesmus* produces additional cell wall material, total polysaccharides, which connect cells in the colony (Trainor 1998; Yang et al. 2007). The surrounding cell wall of *Chlorella* and *Scendesmus* colonies is usually composed of a series of polysaccharide sporopollenin-containing layers, the outer layer being most often mucilaginous and invisible (Round 1965; Atkinson et al. 1972; Pickett-Heaps 1975). Future studies on the exact biochemical pathways and the genomic toolkit regulating the synthesis of this cell wall adhesive material would help us for example: (1) measure the cost of making this material by comparing cell-wall-producing and non-producing strains (having knocked-out specific genes involved in cell wall-synthesis); (2) test whether this adhesive material is absolutely necessary for group formation; (3) and examine whether chemically or genetically engineered overexpression of this material makes groups more stable and irreversible over time. For instance, in the Volvocine algae, innovations in genes (e.g. duplications) involved in producing the extracellular matrix, have played a key role in the evolutionary transition from single cells to obligate multicellularity (Merchant et al. 2007;

Prochnik et al. 2010).

Table 6.1. Scanning Electron Microscopy images of algal colonies.

Algae	Algal colony	Figures reproduced from
Chlorella sorokiniana		this study
Chlorella vulgaris		Boraas et al. 1998
Scenedesmus sp.		Qiao et al. 2015
Green alga *Coelastrum*		Marchant 1977

Group formation: additional ultimate explanations

Clump: why reverse?

In our study we see that single cells form groups in response to predators, and this group formation provides a benefit upon exposure to small predators (Chapters 2, 4 & 5). However, over time, groups reverse back to single cells (Chapter 2; Lürling 1998; Fisher et al. 2016), indicating that these multicellular groups are not a major transition, they are not an obligately multicellular organism. They are a minor transition in multicellularity, a facultatively multicellular organism. Why do they reverse? This reversal may be explained by: (1) the fitness costs associated with group formation (Chapter 4); and (2) the way in which groups form, both subsocially and semisocially (Chapter 3), where in the latter case conflicts can arise among cells due to the possibility of non-relatives joining the group. Taking this point further, it would be interesting to examine whether groups composed of different algal species break up into single cells faster, i.e. are less stable, over time, presumably due to higher conflict among cells, than groups composed of members of the same algal species.

Why be plastic versus fixed?

C. sorokiniana, C. vulgaris, and *S. obliquus* display the plastic phenotypic response of group formation depending on the presence or absence of predators. Phenotypic plasticity is defined as a single genotype producing multiple phenotypes depending on the environment (Schlichting and Pigliucci 1998; DeWitt and Scheiner 2004). Such facultative group formation in response to predators appears in many taxa across the tree of life (Fig. 6.2; Trainor et al. 1971). But why do algae display such a plastic response and not just a fixed phenotype? Over evolutionary time, such plastic responses are usually favoured when organisms face variable environments (Karban and Baldwin 1997; Agrawal 1998; Tollrian and Harvell 1999; Tollrian and Dodson 1999; Agrawal 2001). Plasticity is often favoured under: (1) high spatial variation; (2) high dispersal; (3) high temporal variability, (4) reliable environmental cues; (5) high genetic variability for plasticity; (6) low costs of plasticity; and (7) when specific phenotypes are optimal under different environmental conditions (Padilla and Adolph 1996; Reed et al. 2010; Hendry 2015). Quoting Darwin (1859), "…it is not the strongest that survive or the most intelligent, but the ones most responsive to change". Algae live in environments of rapid changes in various factors, including predation pressure, at different temporal and spatial scales, thus favouring phenotypic plasticity (Round 1965; Sterner 1989). Future attempts to examine the exact timing/duration of the predatory danger and long-term costs of the inducible defence of group formation, would improve our understanding of the exact conditions favouring such inducible defences versus a fixed phenotype (Lima and Bednekoff 1999).

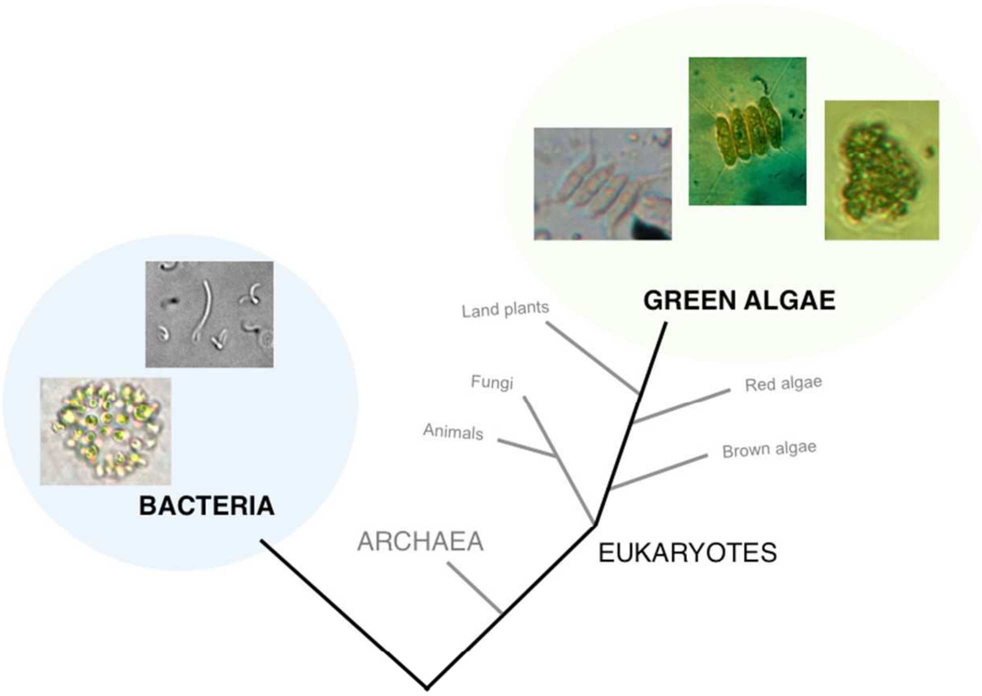


Figure 6.2. Taxa where the facultative formation of multicellular groups, in response to predators, has been studied. Previous studies have examined both bacteria (4

genera, 4 species: *Microcystis, Flectobacillus, Comamonas* and *Cyanobium*), and green algae (6 genera, 13 species: *Scenedesmus, Desmodesmus, Coelastrum, Phaeocystis, Chlamydomonas, Chlorella*) (Hessen and Van Donk 1993; Lürling and Van Donk 1997; Boraas et al. 1998; Van Donk et al. 1999; Von Elert and Franck 1999; Hahn et al. 1999; Lürling 1999; Jakobsen and Tang 2002; Ha et al. 2004; Jezberová and Komárková 2007; Yang et al. 2009; Becks et al. 2010; Fisher et al., 2016). The distribution of these species within those groups suggest multiple independent evolutions of facultative multicellular group formation and/or multiple losses. Images from left to right show: *Microcystis* (https://en.wikipedia.org/wiki/Microcystis#/media/File: Microcystis_aeruginosa.jpeg), *Flectobacillus* (image courtesy of Lorenzo Santorelli), *Desmodesmus* (https://en.wikipedia.org/wiki/Desmodesmus#/media/ File:Desmodesmus-arm-atus-var-armatus.JPG), *Scenedesmus* (https://en.wikipedia.org/wiki/Scenedesmus#/media/File:Scenedesmus_GLERL.jpg) and *Chlorella vulgaris* (courtesy of Stefania Kapsetaki).

What abiotic factors favour multicellularity?

We know quite a lot about the role of relatedness (Hamilton 1964; Maynard Smith and Szathmary 1995; Grosberg and Strathmann 1998; Queller 2000; Michod and Roze 2001; Grosberg and Strathmann 2007; Bourke 2011; Kuzdzal-Fick et al. 2011; Fisher et al. 2013) and ecology, such as the biotic factor of predation (e.g. this study), in social group formation and the transition to obligate multicellularity. But little do we know about the abiotic factors favouring the evolution of multicellularity. Are there any particular abiotic factors favouring a minor or major transition in multicellularity across all the algal taxa on the tree of life? There are hints suggesting three abiotic components favouring multicellularity. First, as mentioned in the previous paragraph, in contrast to variable/fluctuating environments favouring the evolution of unstable reversible multicellular groups, a more stable environment may favour a fixed phenotype, an irreversible obligately multicellular group. Second, low temperatures, as seen in the algae *Scenedesmus,* trigger multicellular group formation (Egan and Trainor 1989; Trainor 1992; Trainor 1993). Third, across the multitude of freshwater, marine, terrestrial, or even a combination of these habitats colonised by algae, Bonner (1998) has suggested that aqueous environments favour multicellular group formation by cells remaining stuck together after division, a mechanism of group formation that has given rise to all the known obligate multicellular organisms on the tree of life. A future comparative project across algae investigating any patterns between such abiotic components - stable, cold, and/or aqueous environments - and multicellular complexity would certainly enhance our understanding of the evolution of multicellularity.

Concluding remarks

This thesis contributes to the fields of evolution and ecology by providing a more solid understanding of multicellular group formation, how and why single cells form groups and the role of predation in inducing the minor transition from single cells to multicellular groups.

References

Aarnio K, Bonsdorff E. 1997. Passing the gut of juvenile flounder, *Platichthys flesus*: differential survival of zoobenthic prey species. Mar. Biol. 129:11–14.

Ackermann M. 2015. A functional perspective on phenotypic heterogeneity in microorganisms. Nat. Rev. Microbiol. 13:497–508.

Agrawal A a. 1998. Algal defense, grazers, and their interactions in aquatic trophic cascades. Acta Oecologica 19:331–337.

Agrawal AA. 2001. Phenotypic plasticity in the interactions and evolution of species. Science (80-.). 294:321–326.

Atkinson jun AW, Gunning BES, John PCL. 1972. Sporopollenin in the cell wall of *Chlorella* and other algae: ultrastructure, chemistry, and incorporation of 14C-acetate, studied in synchronous cultures. Planta 107:1–32.

Baker JR. 1948. The status of the Protozoa. Nature 161:548.

Barlow JP, Bishop JW. 1965. Phosphate regeneration by zooplankton in Cayuga Lake. Limnol. Oceanogr. 10:R15–R24.

Bartholmeé S, Samchyshyna L, Santer B, Lampert W. 2005. Subitaneous eggs of freshwater copepods pass through fish guts: Survival, hatchability, and potential ecological implications. Limnol. Oceanogr. 50:923–929.

Bateson P, Laland KN. 2013. Tinbergen's four questions: an appreciation and an update. Trends Ecol. Evol. 28:712–718.

Beakes GW, Canter HM, Jaworski GHM. 1988. Zoospore ultrastructure of *Zygorhizidium affluens* and *Z. planktonicum*, two chytrids parasitizing the diatom *Asterionella formosa*. Can. J. Bot. 66:1054–1067.

Becks L, Ellner SP, Jones LE, Hairston Jr NG. 2010. Reduction of adaptive genetic diversity radically alters eco-evolutionary community dynamics. Ecol. Lett. 13:989–

997.

Berleman JE, Kirby JR. 2009. Deciphering the hunting strategy of a bacterial wolfpack. FEMS Microbiol. Rev. 33:942–957.

Bern L. 1990. Postcapture particle size selection by *Daphnia cucullata* (Cladocera). Limnol. Oceanogr. 35:923–926.

Biernaskie JM, West SA. 2015. Cooperation, clumping and the evolution of multicellularity. Proc. R. Soc. B Biol. Sci.

Blanken W, Janssen M, Cuaresma M, Libor Z, Bhaiji T, Wijffels RH. 2014. Biofilm growth of *Chlorella sorokiniana* in a rotating biological contactor based photobioreactor. Biotechnol. Bioeng. 111:2436–2445.

Bonner J. 2009. First signals: the evolution of multicellular development. [accessed 2011 May 1].

Bonner JT. 1998. The origins of multicellularity. Integr. Biol. Issues, News, Rev. 1:27–36.

Bonner JT. 2003. Evolution of development in the cellular slime molds. Evol. Dev. 5:305–313.

Bonner JT. 2009. The social amoebae: the biology of cellular slime molds. Princeton University Press.

Boomsma JJ. 2007. Kin selection versus sexual selection: why the ends do not meet. Curr. Biol. 17:R673–R683.

Boomsma JJ. 2009. Lifetime monogamy and the evolution of eusociality. Philos. Trans. R. Soc. B Biol. Sci. 364:3191–3207.

Boraas ME, Seale DB, Boxhorn JE. 1998. Phagotrophy by flagellate selects for colonial prey: A possible origin of multicellularity. Evol. Ecol. 12:153–164.

Boraas ME, Seale DB, Holen D. 1992. Predatory behavior of *Ochromonas* analyzed with video microscopy. Arch. für Hydrobiol. 123:459–468.

Bourke AFG. 2011. Principles of social evolution. Oxford University Press Oxford.

Brönmark C, Hansson L. 2000. Chemical communication in aquatic systems: an introduction. Oikos 88:103–109.

Brown JS, Kotler BP. 2007. Foraging and the ecology of fear. Foraging Behav. Ecol.:437–480.

Cadée GC. 1989. Size-selective transport of shells by birds and its paleoecological implications. Palaeontology 32:429–437.

Cadée GC. 2008. Oystercatchers *Haematopus ostralegus* catching Pacific oysters *Crassostrea gigas*. Basteria 72:25–31.

Chavez-Dozal A, Gorman C, Erken M, Steinberg PD, McDougald D, Nishiguchi MK. 2013. Predation response of Vibrio fischeri biofilms to bacterivorus protists. Appl. Environ. Microbiol. 79:553–558.

Christensen AM. 1970. Feeding biology of the sea star *Astropecten irregularis* Pennant. Ophelia 8:1–134.

Christoffersen K. 1996. Ecological implications of cyanobacterial toxins in aquatic food webs. Phycologia 35:42–50.

Claessen D, Rozen DE, Kuipers OP, Søgaard-Andersen L, van Wezel GP. 2014. Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. Nat. Rev. Microbiol. 12:115–124.

Clare JP. 2002. Daphnia, Aquarist guide. Pennak Robert Freshw. Invertebr. United States Am. 20.

Collins K, Gorovsky MA. 2005. *Tetrahymena thermophila*. Curr. Biol. 15:R317–R318.

Consi TR, Passani MB, Macagno ER. 1990. Eye movements in *Daphnia magna*. J.

Comp. Physiol. A 166:411–420.

Conway DVP, McFadzen IRB, Tranter PRG. 1994. Digestion of copepod eggs by larval turbot *Scophthalmus maximus* and egg viability following gut passage. Mar. Ecol. Prog. Ser. 106:303–309.

Conway K, Trainor FR. 1972. *Scenedesmus* morphology and flotation. J. Phycol. 8:138–143.

Cooper, G. A. & West SA. Division of labour and the evolution of extreme specialisation. Nat. Ecol. Evol. (in press).

Coors A, Vanoverbeke J, De Bie T, De Meester L. 2009. Land use, genetic diversity and toxicant tolerance in natural populations of *Daphnia magna*. Aquat. Toxicol. 95:71–79.

Corno G, Jürgens K. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. Appl. Environ. Microbiol. 72:78–86.

Crawley MJ. 2012. The R book. John Wiley & Sons.

Darch SE, West SA, Winzer K, Diggle SP. 2012. Density-dependent fitness benefits in quorum-sensing bacterial populations. Proc. Natl. Acad. Sci. 109:8259–8263.

Darwin C. 1859. The Origin of Species by Means of Natural Election, Or the Preservation of Favored Races in the Struggle for Life. AL Burt.

Davenport J, Moloney T V, Kelly J. 2011. Common sea anemones *Actinia equina* are predominantly sessile intertidal scavengers. Mar. Ecol. Prog. Ser. 430:147–155.

Davies NB, Krebs JR, West SA. 2012. An introduction to behavioural ecology. John Wiley & Sons.

Dehning I, Tilzer MM. 1989. Survival of *Scenedesmus acuminatus* (Chlorophyceae) in darkness. J. Phycol. 25:509–515.

DeMott WR. 1995. The influence of prey hardness on *Daphnia*'s selectivity for large prey. In: Cladocera as Model Organisms in Biology. Springer. p. 127–138.

DeMott WR, Zhang Q, Carmichael WW. 1991. Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. Limnol. Oceanogr. 36:1346–1357.

Devercelli M, Williner V. 2006. Diatom grazing by *Aegla uruguayana* (Decapoda: Anomura: Aeglidae): digestibility and cell viability after gut passage. In: Annales de Limnologie-International Journal of Limnology. Vol. 42. EDP Sciences. p. 73–77.

DeWitt TJ, Scheiner SM. 2004. Phenotypic plasticity: functional and conceptual approaches. Oxford University Press.

Dicke M, Sabelis MW. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? Funct. Ecol.:131–139.

Dictionaries C. 2009. Collins english dictionary. HarperCollins Publishers.

Dictionary C. 2015. Cambridge dictionaries online.

Dictionary M-W. 2006. The Merriam-Webster Dictionary. Merriam-Webster, Incorporated.

Doerder FP, Arslanyolu M, Saad Y, Kaczmarek M, Mendoza M, Mita B. 1996. Ecological genetics of *Tetrahymena thermophila*: mating types, i-antigens, multiple alleles and epistasis. J. Eukaryot. Microbiol. 43:95–100.

Van Donk E, Ianora A, Vos M. 2011. Induced defences in marine and freshwater phytoplankton: a review. Hydrobiologia 668:3–19.

Van Donk E, Lürling M, Lampert W. 1999. Consumer-induced changes in phytoplankton: inducibility, costs, benefits and the impact on grazers. Princeton University Press.

Dworkin M, Bonner JT. 1972. The myxobacteria: new directions in studies of procaryotic development. CRC Crit. Rev. Microbiol. 1:435–452.

Ebert D. 2005. Ecology, epidemiology, and evolution of parasitism in *Daphnia*. National Library of Medicine.

Egan PF, Trainor FR. 1989. Low cell density: the unifying principle for unicell development in *Scenedesmus* (Chlorophyceae). Br. Phycol. J. 24:271–283. [accessed 2014 Nov 2].

Von Elert E, Franck A. 1999. Colony formation in *Scenedesmus*: grazer-mediated release and chemical features of the infochemical. J. Plankton Res. 21:789–804.

Epp RW, Lewis WM. 1981. Photosynthesis in copepods. Science (80-.). 214:1349–1350.

Fenchel T, Kinne O. 1987. Ecology: Potentials and limitations. Ecology Institute Oldendorf/Luhe.

Figuerola J, Green AJ. 2002. Dispersal of aquatic organisms by waterbirds: a review of past research and priorities for future studies. Freshw. Biol. 47:483–494.

Fisher RM, Bell T, West SA. 2016. Multicellular group formation in response to predators in the alga *Chlorella vulgaris*. J. Evol. Biol. 29:551–559.

Fisher RM, Cornwallis CK, West SA. 2013. Group formation, relatedness, and the evolution of multicellularity. Curr. Biol. 23:1120–1125.

Flinkman J, Vuorinen I, Christiansen M. 1994. Calanoid copepod eggs survive passage through fish digestive tracts. ICES J. Mar. Sci. 51:127–129.

Friman V-P, Diggle SP, Buckling A. 2013. Protist predation can favour cooperation within bacterial species. Biol. Lett. 9:20130548.

Gavrilets S. 2010. Rapid transition towards the division of labor via evolution of developmental plasticity. PLoS Comput Biol 6:e1000805–e1000805.

Gladyshev MI, Kolmakov VI, Dubovskaya OP, Ivanova EA. 2000. The microalgal food spectrum of *Daphnia longispina* during the algal bloom of an eutrophic water body. In: Doklady Biological Sciences Section C/C Of Doklady-Akademiia Nauk Sssr. Vol. 371. Nauka/Interperiodica Publishing. P. 179–181.

Gliwicz MZ. 1986. Predation and the evolution of vertical migration in zooplankton. Nature 320:746.

Green RF, Gordh G, Hawkins BA. 1982. Precise sex ratios in highly inbred parasitic wasps. Am. Nat. 120:653–665.

Grosberg RK, Strathmann RR. 1998. One cell, two cell, red cell, blue cell: the persistence of a unicellular stage in multicellular life histories. Trends Ecol. Evol. 13:112–116.

Grosberg RK, Strathmann RR. 2007. The Evolution of Multicellularity: A Minor Major Transition? Annu. Rev. Ecol. Evol. Syst. 38:621–654.

Gutzeit G, Lorch D, Weber A, Engels M, Neis U. 2005. Bioflocculent algal–bacterial biomass improves low-cost wastewater treatment. Water Sci. Technol. 52:9–18.

Ha K, Jang M-H, Takamura N. 2004. Colony formation in planktonic algae induced by zooplankton culture media filtrate. J. Freshw. Ecol. 19:9–16.

Hahn MW, Moore ERB, Höfle MG. 1999. Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Appl. Environ. Microbiol. 65:25–35.

Hamilton WD. 1964. The genetical evolution of social behaviour. I. J. Theor. Biol. 7:1–16.

Haney JF. 1973. An in situ examination of the grazing activities of natural zooplankton communities. Arch. fur Hydrobiol. 72:87–132.

Hansen SK, Haagensen JAJ, Gjermansen M, Jørgensen TM, Tolker-Nielsen T, Molin S. 2007. Characterization of a *Pseudomonas putida* rough variant evolved in a mixed-species biofilm with *Acinetobacter* sp. strain C6. J. Bacteriol. 189:4932–4943.

Hanski I, Ranta E. 1983. Coexistence in a patchy environment: three species of *Daphnia* in rock pools. J. Anim. Ecol.:263–279.

Harris EH. 2009. The *Chlamydomonas* sourcebook: introduction to *Chlamydomonas* and its laboratory use. Academic Press.

Hartmann HJ. 1985. Feeding of *Daphnia pulicaria* and *Diaptomus ashlandi* on mixtures of unicellular and filamentous algae. Verh. Int. Verein. Limnol 22:3178–3183.

Hartmann HJ, Kunkel DD. 1991. Mechanisms of food selection in *Daphnia*. In: Biology of Cladocera. Springer. p. 129–154.

Hedges L V, Gurevitch J, Curtis PS. 1999. The meta-analysis of response ratios in experimental ecology. Ecology 80:1150–1156.

Hendry AP. 2015. Key questions on the role of phenotypic plasticity in eco-evolutionary dynamics. J. Hered. 107:25–41.

Herron MD, Borin JM, Boswell JC, Walker J, Knox CA, Boyd M, Rosenzweig F, Ratcliff WC. 2018. De novo origin of multicellularity in response to predation. bioRxiv:247361.

Hessen DO, Van Donk E. 1993. Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. Arch. für Hydrobiol. 127:129–140.

Hoek C, Van den Hoeck H, Mann D, Jahns HM. 1995. Algae: an introduction to phycology. Cambridge university press.

van Holthoon FL, Van Beek TA, Lürling M, Van Donk E, De Groot A. 2003. Colony formation in *Scenedesmus*: a literature overview and further steps towards the chemical characterisation of the *Daphnia* kairomone. Hydrobiologia 491:241–254.

Horn MH. 1997. Evidence for dispersal of fig seeds by the fruit-eating characid fish

Brycon guatemalensis Regan in a Costa Rican tropical rain forest. Oecologia 109:259–264.

Hornby AS. 1974. Oxford dictionary. Oxford Advanced Learner's Dictionary of Current English.

Jakobsen H, Tang K. 2002. Effects of protozoan grazing on colony formation in *Phaeocystis globosa* (Prymnesiophyceae) and the potential costs and benefits. Aquat. Microb. Ecol. 27:261–273.

Jarnagin ST, Kerfoot WC, Swan BK. 2004. Zooplankton life cycles: Direct documentation of pelagic births and deaths relative to diapausing egg production. Limnol. Oceanogr. 49:1317–1332.

Jarnagin ST, Swan BK, Kerfoot WC. 2000. Fish as vectors in the dispersal of *Bythotrephes cederstroemi*: diapausing eggs survive passage through the gut. Freshw. Biol. 43:579–589.

Jezberová J, Komárková J. 2007. Morphological transformation in a freshwater *Cyanobium* sp. induced by grazers. Environ. Microbiol. 9:1858–1862.

Johnson MB, Wen Z. 2010. Development of an attached microalgal growth system for biofuel production. Appl. Microbiol. Biotechnol. 85:525–534.

Kampe H, König-Rinke M, Petzoldt T, Benndorf J. 2007. Direct effects of *Daphnia*-grazing, not infochemicals, mediate a shift towards large inedible colonies of the gelatinous green alga *Sphaerocystis schroeteri*. Limnol. Manag. Inl. Waters 37:137–145.

Kapsetaki SE, West SA. The costs and benefits of multicellular group formation in algae. (Submitted at *Evolution*)

Kapsetaki SE, Fisher RM, West SA. 2016. Predation and the formation of multicellular groups in algae. Evol. Ecol. Res. 17:651–669.

Kapsetaki SE, Tep A, West SA. 2017. How do algae form multicellular groups? Evol. Ecol. Res. 18:663–675.

Karban R, Baldwin IT. 1997. Induced defense and evolution of induced resistance. Induc. Responses to Herbiv.:167–204.

Kaushik S, Katoch B, Nanjundiah V. 2006. Social behaviour in genetically heterogeneous groups of *Dictyostelium giganteum*. Behav. Ecol. Sociobiol. 59:521–530.

Kerszberg M, Wolpert L. 1998. The origin of metazoa and the egg: a role for cell death. J. Theor. Biol. 193:535–537.

Kim DH, Casale D, Kőhidai L, Kim MJ. 2009. Galvanotactic and phototactic control of *Tetrahymena pyriformis* as a microfluidic workhorse. Appl. Phys. Lett. 94:163901.

Kiørboe T, Andersen KP, Dam HG. 1990. Coagulation efficiency and aggregate formation in marine phytoplankton. Mar. Biol. 107:235–245.

Kiørboe T, Lundsgaard C, Olesen M, Hansen JLS. 1994. Aggregation and sedimentation processes during a spring phytoplankton bloom: A field experiment to test coagulation theory. J. Mar. Res. 52:297–323.

Kirk DL. 2005. A twelve-step program for evolving multicellularity and a division of labor. Bioessays 27:299–310.

Kirk JTO. 1994. Light and photosynthesis in aquatic ecosystems. Cambridge university press.

Knisely K, Geller W. 1986. Selective feeding of four zooplankton species on natural lake phytoplankton. Oecologia 69:86–94.

Knoll AH. 2003. Biomineralization and evolutionary history. Rev. Mineral. geochemistry 54:329–356.

Knoll AH. 2011. The multiple origins of complex multicellularity. Annu. Rev. Earth

Planet. Sci. 39:217–239.

Koschwanez JH, Foster KR, Murray AW. 2011. Sucrose utilization in budding yeast as a model for the origin of undifferentiated multicellularity. PLoS Biol. 9:e1001122.

Koschwanez JH, Foster KR, Murray AW. 2013. Improved use of a public good selects for the evolution of undifferentiated multicellularity. Elife 2:e00367.

Kretzschmar M, Nisbet RM, McCauley E. 1993. A predator-prey model for zooplankton grazing on competing algal populations. Theor. Popul. Biol. 44:32–66.

Krienitz L, Huss VAR, Bock C. 2015. *Chlorella*: 125 years of the green survivalist. Trends Plant Sci. 20:67–69.

Kruuk H. 2003. Niko's nature: the life of Niko Tinbergen and his science of animal behaviour. OUP Oxford.

Kuzdzal-Fick JJ, Fox SA, Strassmann JE, Queller DC. 2011. High relatedness is necessary and sufficient to maintain multicellularity in *Dictyostelium*. Science (80-.). 334:1548–1551.

Kylin A, Das G. 1967. Calcium and strontium as micronutrients and morphogenetic factors for *Scenedesmus*. Phycologia 6:201–210.

Lampert W. 1987. Feeding and nutrition in *Daphnia*. Mem. Ist. Ital. Idrobiol 45:143–192.

Lampert W, Rothhaupt KO, Elert E Von. 1994. Chemical induction of colony formation in a green alga (*Scenedesmus acutus*) by grazers (*Daphnia*). Limnol. Oceanogr. 39:1543–1550.

Lancelot C, Mathot S. 1985. Biochemical fractionation of primary production by phytoplankton in Belgian coastal waters during short-and long-term incubations with 14C-bicarbonate. Mar. Biol. 86:227–232.

Lancelot C, Rousseau V, Green JC, Leadbeater BSC. 1994. Ecology of *Phaeocystis*-

dominated ecosystems: the key role of colony forms. The Haptophyte Algae:229–245.

Lass S, Spaak P. 2003. Chemically induced anti-predator defences in plankton: a review. Hydrobiologia 491:221–239.

Laux J, Koelsch G. 2014. Potential for passive internal dispersal: eggs of an aquatic leaf beetle survive passage through the digestive system of mallards. Ecol. Entomol. 39:391–394.

Lee J, Cho D-H, Ramanan R, Kim B-H, Oh H-M, Kim H-S. 2013. Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*. Bioresour. Technol. 131:195–201.

van Leeuwen CHA, van der Velde G. 2012. Prerequisites for flying snails: external transport potential of aquatic snails by waterbirds. Freshw. Sci. 31:963–972.

van Leeuwen CHA, Van der Velde G, van Groenendael JM, Klaassen M. 2012. Gut travellers: internal dispersal of aquatic organisms by waterfowl. J. Biogeogr. 39:2031–2040.

Van Leeuwen CHA, Van Der Velde G, Van Lith B, Klaassen M. 2012. Experimental quantification of long distance dispersal potential of aquatic snails in the gut of migratory birds. PLoS One 7:e32292.

Lima SL, Bednekoff PA. 1999. Temporal variation in danger drives antipredator behavior: the predation risk allocation hypothesis. Am. Nat. 153:649–659.

Long JD, Smalley GW, Barsby T, Anderson JT, Hay ME. 2007. Chemical cues induce consumer-specific defenses in a bloom-forming marine phytoplankton. Proc. Natl. Acad. Sci. U. S. A. 104:10512–10517.

Lopez LCS, Gonçalves DA, Mantovani A, Rios RI. 2002. Bromeliad ostracods pass through amphibian (*Scinaxax perpusillus*) and mammalian guts alive. Hydrobiologia 485:209–211.

Lu T. 1988. A Study on the Growth and Nutrition of a Fresh-water Species of Ochromonas.

Lürling M. 1998. Effect of Grazing-Associated Infochemicals on Growth and Morphological Development in Scenedesmus Acutus (Chlorophyceae. J. Phycol. 586:578–586.

Lürling M. 1999. The smell of water. Grazer-induced Colony Formation in *Scenedesmus*. Agric. Univ. Wageningen.

Lürling M. 2001. Grazing-associated infochemicals induce colony formation in the green alga *Scenedesmus*. Protist 152:7–16.

Lürling M. 2006. Effects of a surfactant (FFD-6) on *Scenedesmus* morphology and growth under different nutrient conditions. Chemosphere 62:1351–1358.

Lürling M. 2011. Metribuzin impairs the unicell-colony transformation in the green alga *Scenedesmus obliquus*. Chemosphere 82:411–417.

Lurling M, Beekman W. 2006. Palmelloids formation in *Chlamydomonas reinhardtii*: defence against rotifer predators? In: Annales de Limnologie-International Journal of Limnology. Vol. 42. EDP Sciences. p. 65–72.

Lürling M, Beekman W. 2002. Extractable substances (anionic surfactants) from membrane filters induce morphological changes in the green alga *Scenedesmus obliquus* (Chlorophyceae). Environ. Toxicol. Chem. 21:1213–1218.

Lürling M, Donk E. 1996. Zooplankton-induced unicell-colony transformation in *Scenedesmus acutus* and its effect on growth of herbivore *Daphnia*. Oecologia 108:432–437.

Lurling M, Van Donk E. 2000. Grazer-induced colony formation in *Scenedesmus*: are there costs to being colonial? Oikos 88:111–118.

Lürling M, Van Donk E. 1997. Morphological changes in *Scenedesmus* induced by infochemicals released in situ from zooplankton grazers. Limnol. Oceanogr. 42:783–788.

Lürling M, De Lange HJ, Van Donk E. 1997. Changes in food quality of the green alga *Scenedesmus* induced by *Daphnia* infochemicals : biochemical composition and morphology. Freshw. Biol. 3:619–628.

Lürling M, Verschoor AM. 2003. FO-spectra of chlorophyll fluorescence for the determination of zooplankton grazing. Hydrobiologia 491:145–157.

Lyons NA, Kolter R. 2015. On the evolution of bacterial multicellularity. Curr. Opin. Microbiol. 24:21–28.

MacGinitie GE. 1939. The method of feeding of *Chaetopterus*. Biol. Bull. 77:115–118.

Mack TN, Andraso G. 2015. Ostracods and other prey survive passage through the gut of round goby (*Neogobius melanostomus*). J. Great Lakes Res. 41:303–306.

Marchant HJ. 1977. Cell Division And Colony Formation In The Green Alga *Coelastrum* (Chlorococcales) 1. J. Phycol. 13:102–110.

Matz C, Deines P, Boenigk J, Arndt H, Eberl L, Kjelleberg S, Ju K, Icrobiol Applenm. 2004. Impact of Violacein-Producing Bacteria on Survival and Feeding of Bacterivorous Nanoflagellates. 70:1593–1599.

Matz C, Kjelleberg S. 2005. Off the hook–how bacteria survive protozoan grazing. Trends Microbiol. 13:302–307.

Maynard Smith J, Szathmary E. 1995. The major transitions in evolution. Oxford; New York: W.H. Freeman Spektrum.

Mayr E. 1961. Cause and effect in biology. Science (80-.). 134:1501–1506.

McClatchie S, Juniper SK, Knox GA. 1982. Structure of a mudflat diatom community in the Avon-Heathcote Estuary, New Zealand. New Zeal. J. Mar. Freshw. Res.

16:299–309.

De Meester L, Dumont HJ. 1988. The genetics of phototaxis in *Daphnia magna*: Existence of three phenotypes for vertical migration among parthenogenetic females. Hydrobiologia 162:47–55.

De Meester L, Dumont HJ. 1989. Phototaxis in *Daphnia*: interaction of hunger and genotype. Limnol. Oceanogr. 34:1322–1325.

Mehdiabadi NJ, Jack CN, Farnham TT, Platt TG, Kalla SE, Shaulsky G, Queller DC, Strassmann JE. 2006. Social evolution: kin preference in a social microbe. Nature 442:881–882.

Meise CJ, Munns Jr WR, Hairston Jr NG. 1985. An analysis of the feeding behavior of *Daphnia pulex* 1. Limnol. Oceanogr. 30:862–870.

Mellors WK. 1975. Selective predation of ephippal *Daphnia* and the resistance of ephippal eggs to digestion. Ecology 56:974–980.

Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ, Witman GB, Terry A, Salamov A, Fritz-Laylin LK, Maréchal-Drouard L. 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science (80-.). 318:245–250.

Michener CD. 1969. Comparative social behavior of bees. Annu. Rev. Entomol. 14:299–342.

Michod RE. 2007. Evolution of individuality during the transition from unicellular to multicellular life. Proc. Natl. Acad. Sci. U. S. A. 104:8613–8618.

Michod RE, Roze D. 2001. Cooperation and connflict in the evolution of multicellularity. Heredity 86:1.

Monahan TJ. 1977. Effects of organic phosphate on the growth and morphology of *Scenedesmus obtusiusculus* (Chlorophyceae). Phycologia 16:133–137.

Monier J-M, Lindow SE. 2005. Spatial organization of dual-species bacterial aggregates on leaf surfaces. Appl. Environ. Microbiol. 71:5484–5493.

Nakajima T, Matsubara T, Ohta Y, Miyake D. 2013. Exploitation or cooperation? Evolution of a host (ciliate)-benefiting alga in a long-term experimental microcosm culture. BioSystems 113:127–139.

Nakajima T, Sano A, Matsuoka H. 2009. Auto-/heterotrophic endosymbiosis evolves in a mature stage of ecosystem development in a microcosm composed of an alga, a bacterium and a ciliate. BioSystems 96:127–135.

Nalepa CA, Piper WH. 1994. Bird dispersal of the larval stage of a seed predator. Oecologia 100:200–202.

Nichols SA, Dayel MJ, King N. 2009. Genomic, phylogenetic, and cell biological insights into metazoan origins. Anim. Evol. Genomes, Foss. trees:24–32.

Nicotri ME. 1977. Grazing effects of four marine intertidal herbivores on the microflora. Ecology 58:1020–1032.

Niklas KJ, Newman SA. 2013. The origins of multicellular organisms. Evol. Dev. 15:41–52.

Nilshammar M, Walles B. 1974. Electron microscope studies on cell differentiation in synchronized cultures of the green alga *Scenedesmus*. Protoplasma 79:317–332.

Norton SF. 1988. Role of the gastropod shell and operculum in inhibiting predation by fishes. Science (80-.). 241:92–94.

O'donnell DR, Fey SB, Cottingham KL. 2012. Nutrient availability influences kairomone-induced defenses in *Scenedesmus acutus* (Chlorophyceae). J. Plankton Res. 35:191–200.

Orias E, Cervantes MD, Hamilton EP. 2011. *Tetrahymena thermophila*, a unicellular eukaryote with separate germline and somatic genomes. Res. Microbiol. 162:578–

586.

Padilla DK, Adolph SC. 1996. Plastic inducible morphologies are not always adaptive: The importance of time delays in a stochastic environment. Evol. Ecol. 10:105–117.

Paine RT. 2011. Food recognition and predation on opistobranchs by *Navanax inermis* (1963). Essent. Nat. Timeless Readings Nat. Hist.:213.

Pajdak-stós A, Fia E, Fyda J. 2001. *Phormidium autumnale* (Cyanobacteria) defense against three ciliate grazer species. 23:237–244.

Pančić M, Kiørboe T. 2018. Phytoplankton defence mechanisms: traits and trade-offs. Biol. Rev. 93:1269–1303.

Peperzak L. 1993. Daily irradiance governs growth rate and colony formation of *Phaeocystis* (Prymnesiophyceae). J. Plankton Res. 15:809–821.

Petersen C, Hermann RJ, Barg M-C, Schalkowski R, Dirksen P, Barbosa C, Schulenburg H. 2015. Travelling at a slug's pace: possible invertebrate vectors of *Caenorhabditis* nematodes. BMC Ecol. 15:19.

Peterson CG. 1987. Gut passage and insect grazer selectivity of lotic diatoms. Freshw. Biol. 18:455–460.

Peterson CG, Boulton AJ. 1999. Stream permanence influences microalgal food availability to grazing tadpoles in arid-zone springs. Oecologia 118:340–352.

De Philippis R, Vincenzini M. 1998. Exocellular polysaccharides from cyanobacteria and their possible applications. FEMS Microbiol. Rev. 22:151–175.

Pickett-Heaps JD. 1975. Green algae: structure, reproduction and evolution in selected genera.

Pietrzak B, Bednarska A, Grzesiuk M. 2010. Longevity of *Daphnia magna* males and females. Hydrobiologia 643:71–75.

Pietrzak B, Grzesiuk M, Bednarska A. 2010. Food quantity shapes life history and survival strategies in *Daphnia magna* (Cladocera). Hydrobiologia 643:51–54.

Ploug H, Stolte W, Jørgensen BB. 1999. Diffusive boundary layers of the colony-forming plankton alga, *Phaeocystis* sp. - implications for nutrient uptake and cellular growth. Limnol. Oceanogr. 44:1959–1967.

Porter KG. 1973. Selective grazing and differential digestion of algae by zooplankton. Nature 244:179.

Porter KG. 1975. Viable gut passage of gelatinous green algae ingested by *Daphnia*: With 1 figure and 5 tables in the text. Int. Vereinigung für Theor. und Angew. Limnol. Verhandlungen 19:2840–2850.

Porter KG. 1976. Enhancement of algal growth and productivity by grazing zooplankton. Science (80-.). 9:1332–1334.

Prochnik SE, Umen J, Nedelcu AM, Hallmann A, Miller SM, Nishii I, Ferris P, Kuo A, Mitros T, Fritz-Laylin LK. 2010. Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. Science (80-.). 329:223–226.

Qiao K, Takano T, Liu S. 2015. Discovery of two novel highly tolerant NaHCO3 Trebouxiophytes: Identification and characterization of microalgae from extreme saline–alkali soil. Algal Res. 9:245–253.

Queck S, Weitere M, Moreno AM, Rice SA, Kjelleberg S. 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ. Microbiol. 8:1017–1025.

Queller DC. 2000. Relatedness and the fraternal major transitions. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 355:1647–1655.

Quijano G, Arcila JS, Buitrón G. 2017. Microalgal-bacterial aggregates: Applications and perspectives for wastewater treatment. Biotechnol. Adv.

R Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raven J. 1998. David L. Kirk. *Volvox*: Molecular-Genetic Origins of Multicellularity and Cellular Differentiation. Developmental and Cell Biology.

Reed TE, Waples RS, Schindler DE, Hard JJ, Kinnison MT. 2010. Phenotypic plasticity and population viability: the importance of environmental predictability. Proc. R. Soc. London B Biol. Sci. 277:3391–3400.

Reynolds CS. 1984. The ecology of freshwater phytoplankton. Cambridge University Press.

Riegman R, Noordeloos AAM, Cadée GC. 1992. Phaeocystis blooms and eutrophication of the continental coastal zones of the North Sea. Mar. Biol. 112:479–484.

Van Rijssel M, Janse I, Noordkamp DJB, Gieskes WWC. 2000. An inventory of factors that affect polysaccharide production by *Phaeocystis globosa*. J. Sea Res. 43:297–306.

Roozen F, Lürling M. 2001. Behavioural response of *Daphnia* to olfactory cues from food, competitors and predators. J. Plankton Res. 23:797–808.

Roper M, Dayel MJ, Pepper RE, Koehl MAR. 2013. Cooperatively generated stresslet flows supply fresh fluid to multicellular choanoflagellate colonies. Phys. Rev. Lett. 110:1–5.

Round FE. 1965. The biology of the algae. New York, St.

Ryther JH. 1954. Inhibitory effects of phytoplankton upon the feeding of *Daphnia magna* with reference to growth, reproduction, and survival. Ecology 35:522–533.

Saint-Jean L, Pagano M. 1995. Egg mortality through predation in egg-carrying zooplankters. Studies on *Heterobranchus longifilis* larvae fed on copepods, cladocerans and rotifers. J. Plankton Res. 17:1501–1512.

Sathe S, Durand PM. 2016. Cellular aggregation in *Chlamydomonas* (Chlorophyceae) is chimaeric and depends on traits like cell size and motility. Eur. J. Phycol. 51:129–138.

Sathe S, Kaushik S, Lalremruata A, Aggarwal RK, Cavender JC, Nanjundiah V. 2010. Genetic heterogeneity in wild isolates of cellular slime mold social groups. Microb. Ecol. 60:137–148.

Sathe S, Khetan N, Nanjundiah V. 2014. Interspecies and intraspecies interactions in social amoebae. J. Evol. Biol. 27:349–362.

Schlichting CD, Pigliucci M. 1998. Phenotypic evolution: a reaction norm perspective. Sinauer Associates Incorporated.

Seale DB, Boraas ME, Holen D, Nealson KH. 1990. Use of biolumescent bacteria, *Xenorhabdus luminescens*, to measure predation on bacteria by freshwater microflagellates. FEMS Microbiol. Ecol. 6:31–39.

Shubert LE, Trainor FR. 1974. *Scenedesmus* morphogenesis. Control of the unicell stage with phosphorus. Br. Phycol. J. 9:1–7.

Siver PA, Trainor FR. 1981. Morphological control and physiology of *Scenedesmus* strain 170. Phycologia 20:1–11.

Sleigh MA. 1989. Adaptations of ciliary systems for the propulsion of water and mucus. Comp. Biochem. Physiol. A. Comp. Physiol. 94:359–364.

Smith J, Queller DC, Strassmann JE. 2014. Fruiting bodies of the social amoeba *Dictyostelium discoideum* increase spore transport by *Drosophila*. BMC Evol. Biol. 14:105.

Solari CA, Galzenati VJ, Kessler JO. 2015. The Evolutionary Ecology of Multicellularity: The Volvocine Green Algae as a Case Study. In: Evolutionary

Transitions to Multicellular Life. Springer. p. 201–223.

Stanley SM. 1973. An Ecological Theory for the Sudden Origin of Multicellular Life in the Late Precambrian. Proc. Natl. Acad. Sci. 70:1486–1489.

Sterner RW. 1989. The role of grazers in phytoplankton succession. In: Plankton ecology. Springer. p. 107–170.

Strassmann JE, Zhu Y, Queller DC. 2000. Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. Nature 408:965–967.

Stutzman P. 1995. Food quality of gelatinous colonial chlorophytes to the freshwater zooplankters *Daphnia pulicaria* and *Diaptomus oregonensis*. Freshw. Biol. 34:149–153.

Szathmáry E, Wolpert L. 2003. The transition from single cells to multicellularity.

Tang KW, Jakobsen HH, Visser AW. 2001. *Phaeocystis globosa* (Prymnesiophyceae) and the planktonic food web: feeding, growth, and trophic interactions among grazers. Limnol. Oceanogr. 46:1860–1870.

Tarnita CE, Taubes CH, Nowak MA. 2013. Evolutionary construction by staying together and coming together. J. Theor. Biol. 320:10–22.

Teyssèdre B. 2006. Are the green algae (phylum Viridiplantae) two billion years old? Carnets Geol.:1–15.

Thornton DCO. 2002. Diatom aggregation in the sea: mechanisms and ecological implications. Eur. J. Phycol. 37:149–161.

Tinbergen N. 1963. On aims and methods of ethology. Z. Tierpsychol. 20:410–433.

Tollrian R, Dodson SI. 1999. Inducible defences in cladocera: constraints, costs, and multipredator environments. Ecol. Evol. inducible defenses (ed TRHCD):177–202.

Tollrian R, Harvell CD. 1999. The ecology and evolution of inducible defenses. Princeton University Press.

Trainor FR. 1992. Cyclomorphosis in *Scenedesmus armatus* (Chlorophyta): An Ordered Sequence of Ecomorph Development 1. J. Phycol. 28:553–558.

Trainor FR. 1993. Cyclomorphosis in *Scenedesmus subspicatus* (Chlorococcales, Chlorophyta): stimulation of colony development at low temperature. Phycologia 32:429–433.

Trainor FR. 1998. Biological aspects of *Scenedesmus* (Chlorophyceae)-phenotypic plasticity. J. Cramer.

Trainor FR, Cain JR, Shubert LE. 1976. Morphology and nutrition of the colonial green alga *Scenedesmus*: 80 years later. Bot. Rev. 42:5–25.

Trainor FR, Rowland HL, Lylis JC, Winter PA, Bonanomi PL. 1971. Some examples of polymorphism in algae. Phycologia 10:113–119.

Tukaj Z, Bohdanowicz J. 1995. Diesel-fuel-oil induced morphological changes in some *Scenedesmus* species (Chlorococcales). Arch. fur Hydrobiol 108:83–94.

Turner JT, Ferrante JG. 1979. Zooplankton fecal pellets in aquatic ecosystems. Bioscience 29:670–677.

Uchida H, Yasumoto K, Nishigami A, Zweigenbaum J a., Kusumi T, Ooi T. 2008. Time-of-flight LC/MS identification and confirmation of a kairomone in *Daphnia magna* cultured medium. Bull. Chem. Soc. Jpn. 81:298–300.

Umen JG. 2014. Green algae and the origins of multicellularity in the plant kingdom. Cold Spring Harb. Perspect. Biol. 6:a016170.

Underwood GJC, Thomas JD. 1990. Grazing interactions between pulmonate snails and epiphytic algae and bacteria. Freshw. Biol. 23:505–522.

Vanni MJ, Lampert W. 1992. Food quality effects on life history traits and fitness in the generalist herbivore *Daphnia*. Oecologia 92:48–57.

Velicer GJ, Vos M. 2009. Sociobiology of the myxobacteria. Annu. Rev. Microbiol.

63:599–623.

Velicer GJ, Yuen-tsu NY. 2003. Evolution of novel cooperative swarming in the bacterium *Myxococcus xanthus*. Nature 425:75–78.

Verschoor AM, Bekmezci OK, van Donk E, Vijverberg J. 2009. The ghost of herbivory past: Slow defence relaxation in the chlorophyte *Scenedesmus obliquus*. J. Limnol. 68:327–335.

Verschoor AM, Zadereev YS, Mooij WM. 2007. Infochemical-mediated trophic interactions between the rotifer *Brachionus calyciflorus* and its food algae. Limnol. Oceanogr. 52:2109–2119.

Vet LEM, Dicke M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. Annu. Rev. Entomol. 37:141–172.

Viechtbauer W. 2010. Conducting meta-analyses in R with the metafor package. J Stat Softw 36:1–48.

Vinyard G. 1979. An ostracod (*Cypriodopsis vidua*) can reduce predation from fish by resisting digestion. Am. Midl. Nat.:188–190.

Wada S, Kawakami K, Chiba S. 2012. Snails can survive passage through a bird's digestive system. J. Biogeogr. 39:69–73.

Wang X, Wang Y, Ou L. 2014. The roles of light–dark cycles in the growth of *Phaeocystis globosa* from the South China Sea: The cost of colony enlargement. J. sea Res. 85:518–523.

Wang X, Wang Y, Ou L, He X, Chen D. 2015. Allocation costs associated with induced defense in *Phaeocystis globosa* (Prymnesiophyceae): the effects of nutrient availability. Sci. Rep. 5:10850.

Weisse T, Scheffel-Möser U. 1990. Growth and grazing loss rates in single-celled *Phaeocystis* sp.(Prymnesiophyceae). Mar. Biol. 106:153–158.

West S, Fisher RM, Gardner A, Kiers ET. 2015. Major evolutionary transitions in individuality. Proc. Natl. Acad. Sci. U. S. A. 112:10112–10119.

West SA, Cooper GA. 2016. Division of labour in microorganisms: an evolutionary perspective. Nat. Rev. Microbiol. 14:716.

Whitton BA, Brook AJ, John DM. 2002. The freshwater algal flora of the British Isles: An identification guide to freshwater and terrestrial algae. Cambridge University Press.

Wiltshire KH, Lampert W, Planck M, Box PO. 1999. Urea excretion by *Daphnia* : A colony-inducing factor in Scenedesmus ? 44:1894–1903.

Wisenden BD. 2000. Olfactory assessment of predation risk in the aquatic environment. Philos. Trans. R. Soc. London B Biol. Sci. 355:1205–1208.

Yamamoto M, Fujishita M, Hirata A, Kawano S. 2004. Regeneration and maturation of daughter cell walls in the autospore-forming green alga *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae). J. Plant Res. 117:257–264.

Yamamoto M, Kurihara I, Kawano S. 2005. Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae). Planta 221:766–775.

Yang Z, Kong F, Shi X, Xing P, Zhang M. 2007. Effects of *Daphnia*-Associated Infochemicals on the Morphology, Polysaccharides Content and PSII-Efficiency in *Scenedesmus obliquus*. Int. Rev. Hydrobiol. 92:618–625.

Yang Z, Kong F, Yang Z, Zhang M, Yu Y, Qian S. 2009. Benefits and costs of the grazer-induced colony formation in *Microcystis aeruginosa*. Ann. Limnol. - Int. J. Limnol. 45:203–208.

Yasumoto K, Nishigami A, Kasai F, Kusumi T, Ooi T. 2006. Isolation and absolute configuration determination of aliphatic sulfates as the *Daphnia* kairomones inducing morphological defense of a phytoplankton. Chem. Pharm. Bull. 54:271–274.

Yasumoto K, Nishigami A, Yasumoto M, Kasai F, Okada Y, Kusumi T, Ooi T. 2005. Aliphatic sulfates released from *Daphnia* induce morphological defense of phytoplankton: Isolation and synthesis of kairomones. Tetrahedron Lett. 46:4765–4767.

Young S. 1974. Directional differences in the colour sensitivity of *Daphnia magna*. J. Exp. Biol. 61:261–267.

Zhang J, Del Aguila R, Schneider C, Schneider BL. 2005. The importance of being big. Journal of Investigative Dermatology Symposium Proceedings. Vol. 10. Elsevier. p. 131–141.

Zhu X, Wang J, Chen Q, Chen G, Huang Y, Yang Z. 2016. Costs and trade-offs of grazer-induced defenses in Scenedesmus under deficient resource. Sci. Rep. 6:22594.

Zhu X, Yang J, Xu N, Chen G, Yang Z. 2015. Combined effects of nitrogen levels and Daphnia culture filtrate on colony size of *Scenedesmus obliquus*. Algal Res. 9:94–98.

Appendix

Evolutionary Ecology Research, 2016, **17**: 651–669

Predation and the formation of multicellular groups in algae

Stefania E. Kapsetaki, Roberta M. Fisher and Stuart A. West

Department of Zoology, University of Oxford, Oxford, UK

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).

Correspondence: S.E. Kapsetaki, Department of Zoology, University of Oxford, South Parks Road, The Tinbergen Building, Oxford OX1 3PS, UK. email: stefania.kapsetaki@zoo.ox.ac.uk

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 ~10^6 cells $\cdot mL^{-1}$.

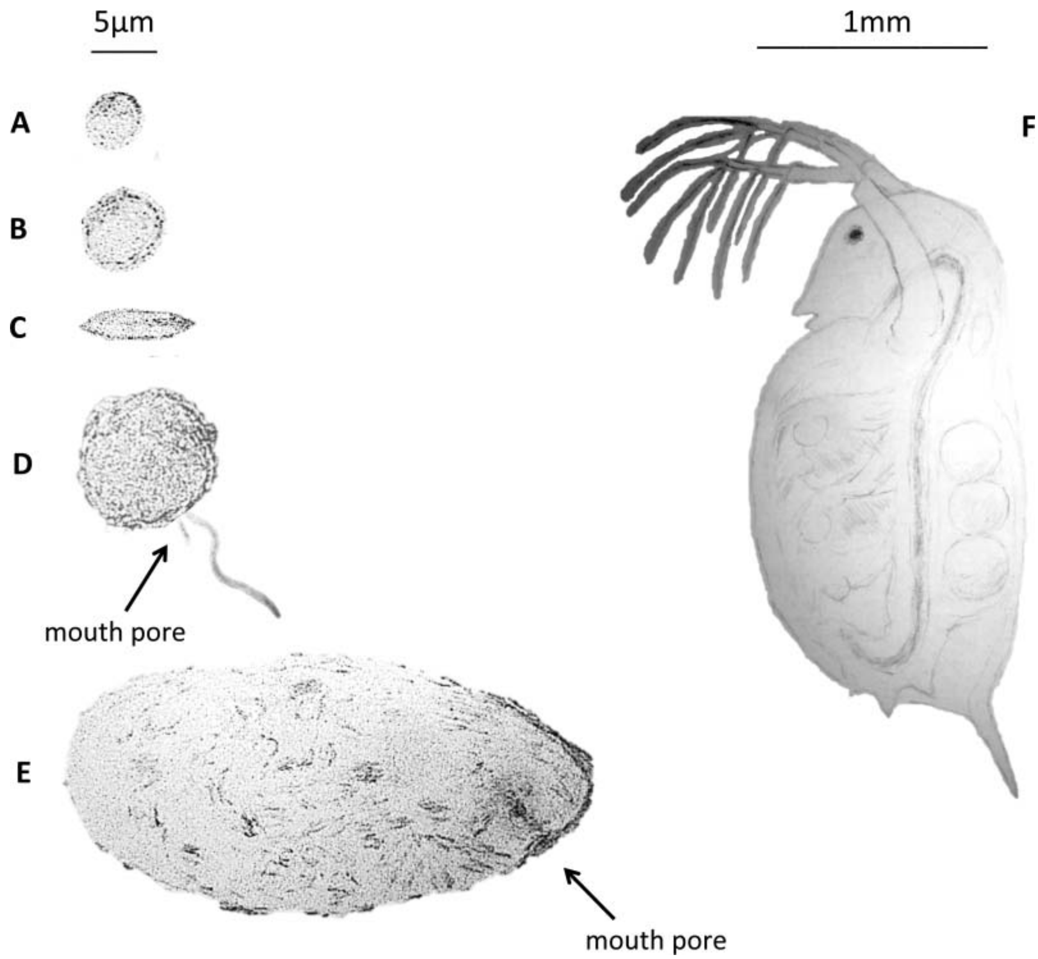


Fig. 1. The species used in the present study: (A) *Chlorella sorokiniana*, (B) *Chlorella vulgaris*, (C) *Scenedesmus obliquus*, (D) the flagellate *Ochromonas*, (E) the ciliate *Tetrahymena thermophila*, and (F) the crustacean *Daphnia magna*.

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	9×10^4	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%

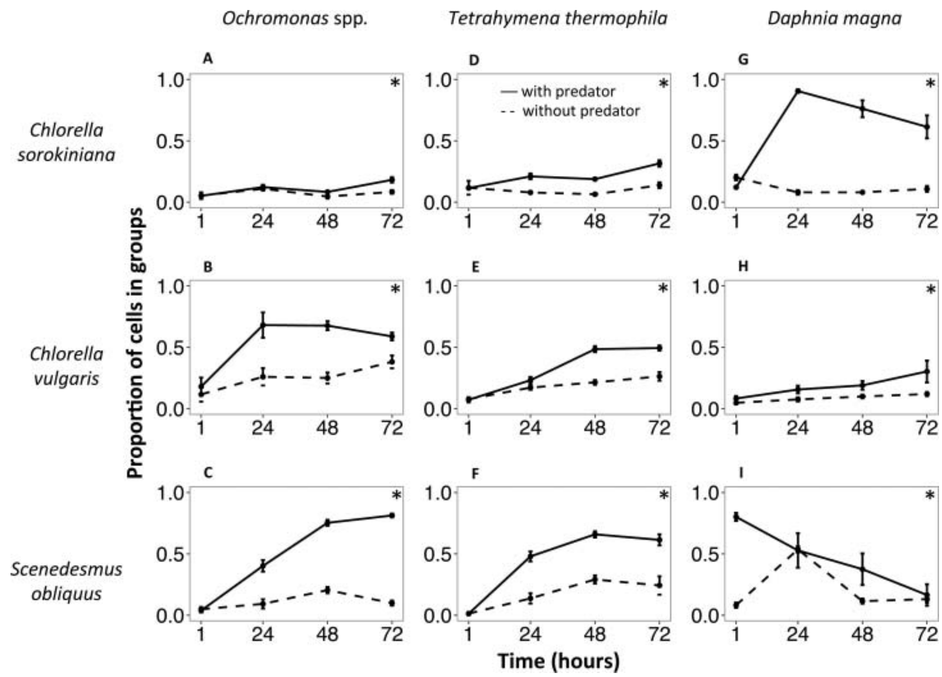


Fig. 2. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In all nine combinations, the proportion of cells in groups was higher in the presence of the putative predator [A: generalized linear model (glm), $F = 11.93$, $P < 0.01$; 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).

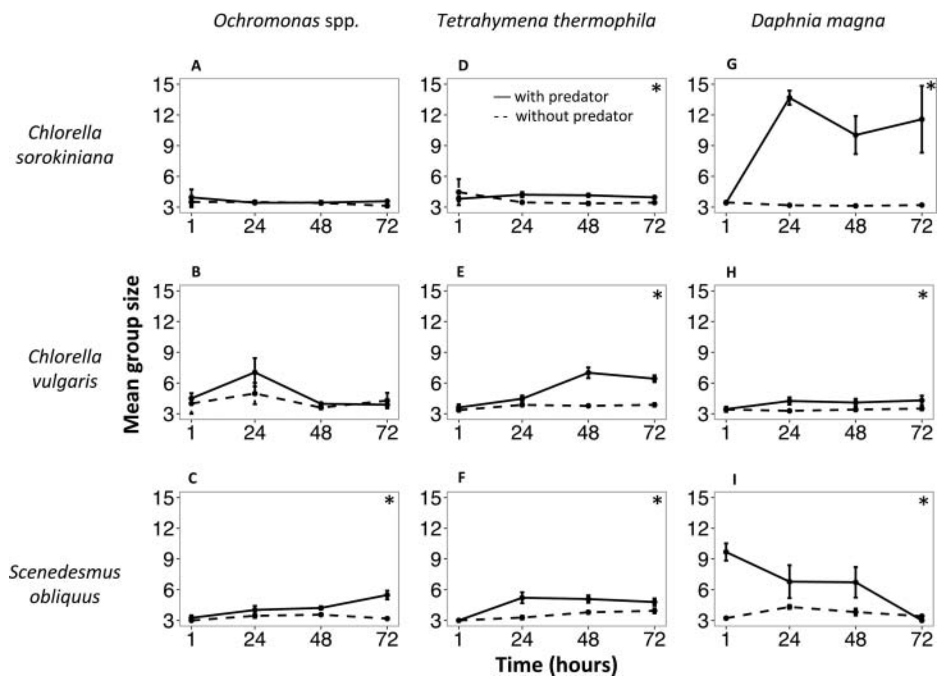


Fig. 3. Mean group size plotted against time in the presence (solid line) and absence (dashed line) of the putative predator. In seven combinations, the mean algal group size was higher in the presence of the putative predator (C: glmmPQL, $P < 0.0001$; D: glm, $F = 11.48$, $P < 0.01$; E: glm, $F = 63.39$, $P < 0.0001$; 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.

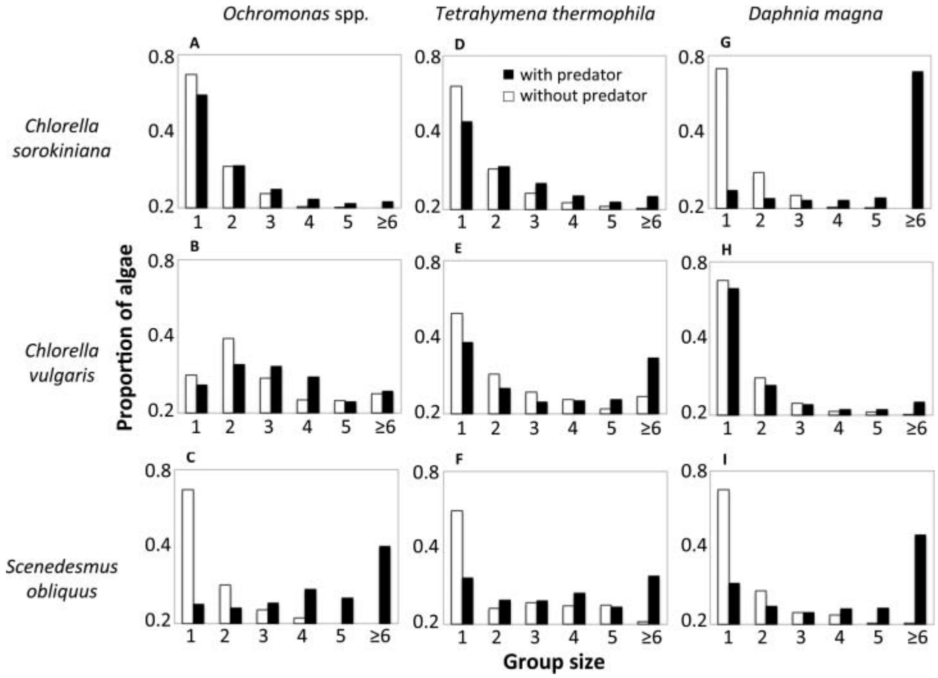


Fig. 4. Distribution of group sizes in the presence (solid bars) and absence (open bars) of the putative predator after 72 hours (A–F) and 48 hours (G–I). Group sizes '1' and '2' refer to a unicell and a paired cell, respectively.

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

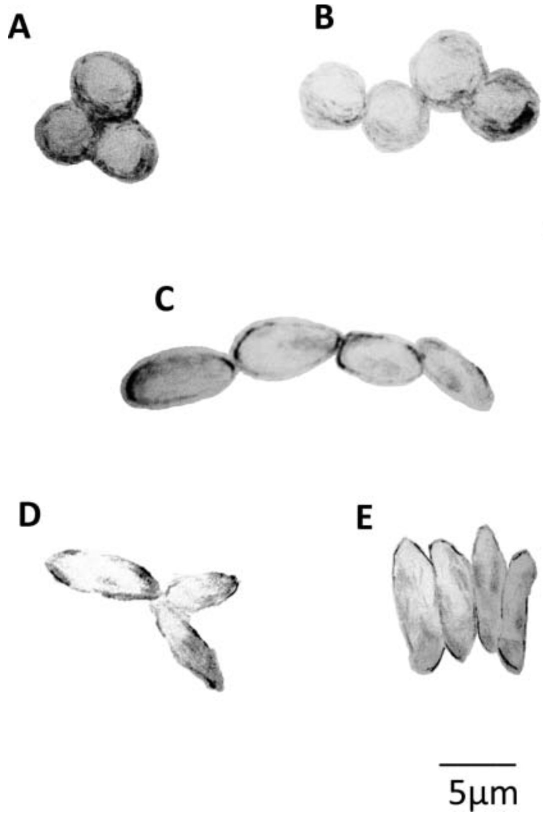


Fig. 5. Characteristic algal group types: (A) three-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (B) four-celled group observed in *C. sorokiniana* and *C. vulgaris* cultures; (D) three-celled group observed in *S. obliquus* cultures; (C, E) four-celled groups seen in *S. obliquus*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

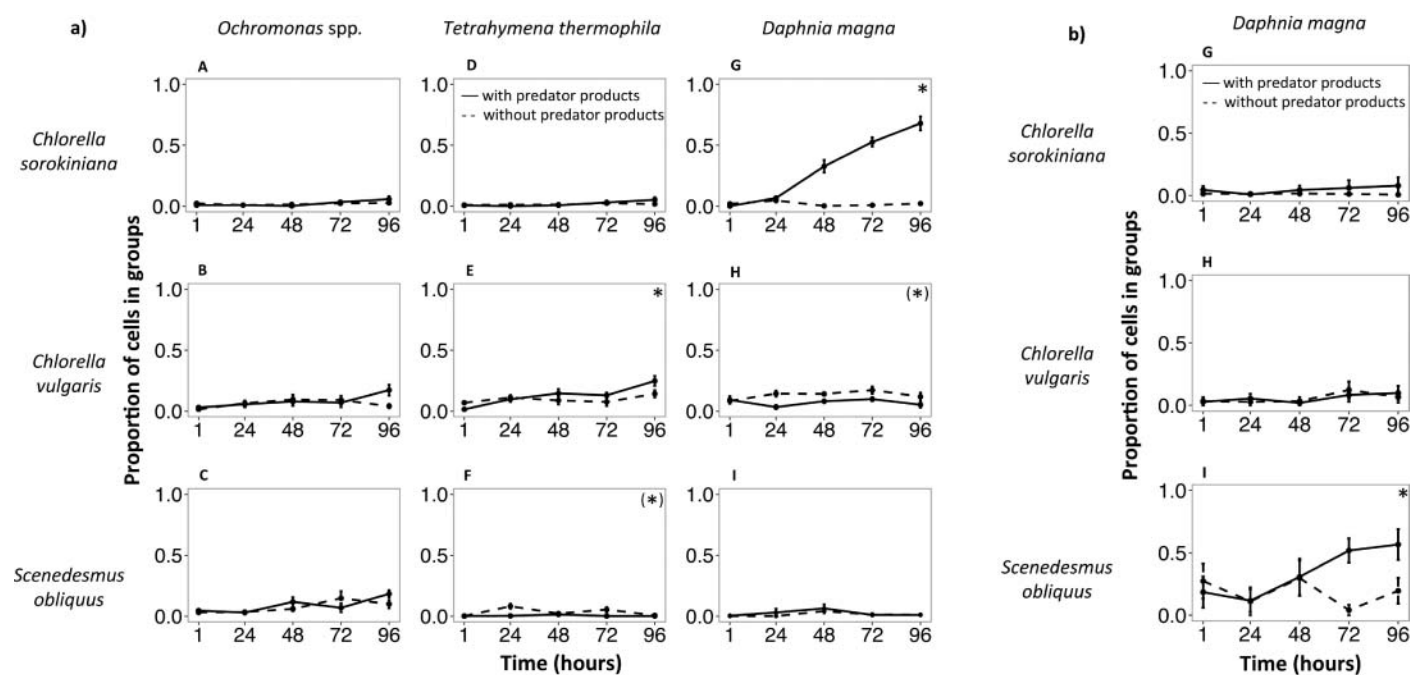


Fig. 6. Proportion of cells in groups plotted against time in the presence (solid line) and absence (dashed line) of predator products. (a) Experiment in tube cultures. In two combinations, the proportion of cells in groups was higher in the presence of the predator products (glmmPQL, E: $P < 0.01$; 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.

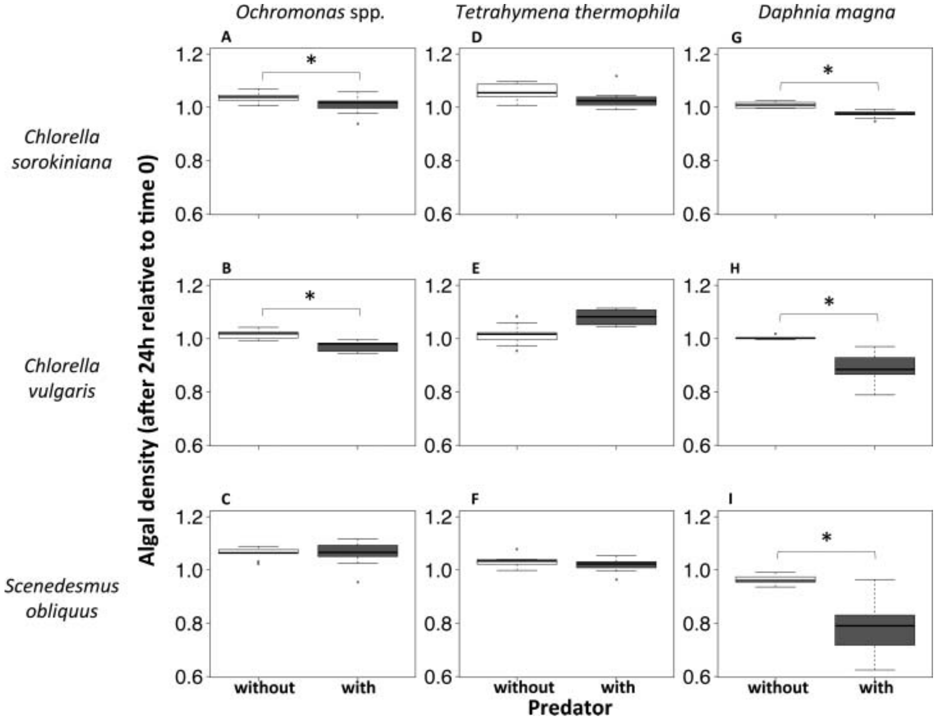


Fig. 7. Algal density (after 24 hours relative to time 0) in the presence (grey) and absence (white) of the putative predator. In five combinations, the algal density decreased in the presence of the putative predator (two-sample *t*-test, A: d.f. = 16, $P = 0.041$; B: d.f. = 16, $P < 0.0001$; G: d.f. = 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,

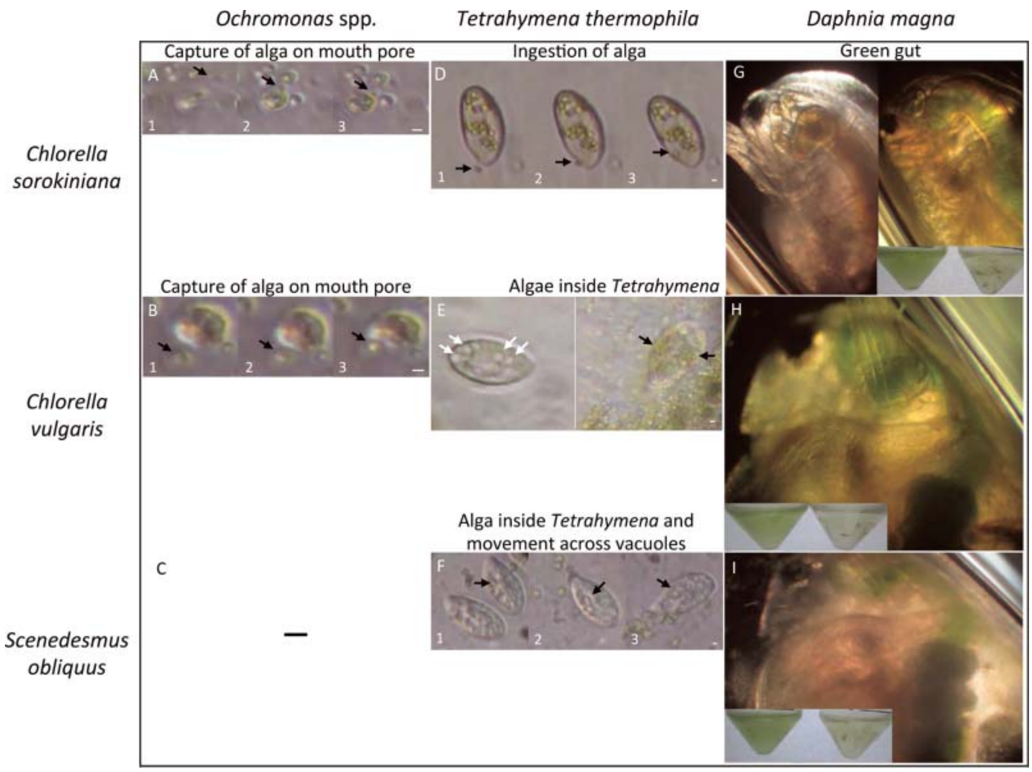


Fig. 8

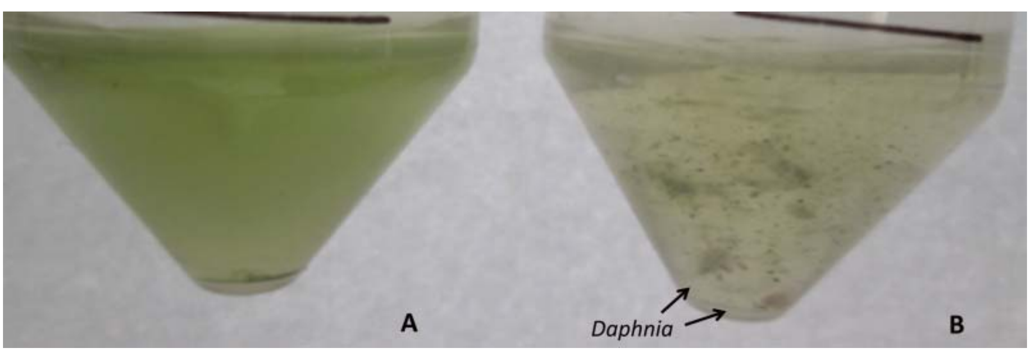


Fig. 9

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).

ACKNOWLEDGEMENTS

We thank Ville Friman, Tom Bell, and Lorenzo Santorelli for supplying organisms; and Andrew Beckerman, Tom Bell, Max Burton, Melanie Ghoul, Ashleigh Griffin, Karl Heilbron, Nella Roccuzzo, Matteo Tanadini, and Lindsay Turnbull for useful comments. This work was supported by the European Research Council (to S.A.W.), the Natural Environment Research Council (to R.M.F.), and the State Scholarships Foundation of Greece, the Alexander S. Onassis Public Benefit Foundation Scholarship, and A.G. Leventis Foundation Scholarship (to S.E.K.).

DATA ACCESSIBILITY

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

REFERENCES

Berleman, J.E. and Kirby, J.R. 2009. Deciphering the hunting strategy of a bacterial wolfpack. *FEMS Microbiol. Rev.*, **33**: 942–957.

Biernaskie, J.M. and West, S.A. 2015. Cooperation, clumping and the evolution of multicellularity. *Proc. R. Soc. Lond. B: Biol. Sci.*, **282**: 20151075.

Boraas, M.E., Seale, D.B. and Holen, D. 1992. Predatory behavior of *Ochromonas* analyzed with video microscopy. *Arch. für Hydrobiol.*, **123**: 459–468.

Boraas, M.E., Seale, D.B. and Boxhorn, J.E. 1998. Phagotrophy by a flagellate selects for colonial prey: a possible origin of multicellularity. *Evol. Ecol.*, **12**: 153–164.

Bourke, A.F.G. 2011. *Principles of Social Evolution*. Oxford: Oxford University Press.

Claessen, D., Rozen, D.E., Kuipers, O.P., Søgaard-Andersen, L. and van Wezel, G.P. 2014. Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat. Rev. Microbiol.*, **12**: 115–124.

Corno, G. and Jürgens, K. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Appl. Environ. Microbiol.*, **72**: 78–86.

Davies, N.B., Krebs, J.R. and West, S.A. 2012. *An Introduction to Behavioural Ecology* (4th edn.). Oxford: Wiley-Blackwell.

Dworkin, M. and Bonner, J.T. 1972. The myxobacteria: new directions in studies of procaryotic development. *CRC Crit. Rev. Microbiol.*, **1**: 435–452.

Fisher, R.M., Cornwallis, C.K. and West, S.A. 2013. Group formation, relatedness, and the evolution of multicellularity. *Curr. Biol.*, **23**: 1120–1125.

Fisher, R.M., Bell, T. and West, S.A. 2016. Multicellular group formation in response to predators in the alga *Chlorella vulgaris*. *J. Evol. Biol.*, **29**: 551–559.

Grosberg, R.K. and Strathmann, R.R. 2007. The evolution of multicellularity: a minor major transition? *Annu. Rev. Ecol. Evol. Syst.*, **38**: 621–654.

Kampe, H., König-Rinke, M., Petzoldt, T. and Benndorf, J. 2007. Direct effects of *Daphnia*-grazing, not infochemicals, mediate a shift towards large inedible colonies of the gelatinous green alga *Sphaerocystis schroeteri*. *Limnologica*, **37**: 137–145.

Kerszberg, M. and Wolpert, L. 1998. The origin of metazoa and the egg: a role for cell death. *J. Theor. Biol.*, **193**: 535–537.

Koschwanez, J.H., Foster, K.R. and Murray, A.W. 2011. Sucrose utilization in budding yeast as a model for the origin of undifferentiated multicellularity. *PLoS Biol.*, **9**: e1001122.

Koschwanez, J.H., Foster, K.R. and Murray, A.W. 2013. Improved use of a public good selects for the evolution of undifferentiated multicellularity. *Elife*, **2**: e00367.

Lampert, W., Rothhaupt, K.O. and Von Elert, E. 1994. Chemical induction of colony formation in a green alga (*Scenedesmus acutus*) by grazers (*Daphnia*). *Limnol. Oceanogr.*, **39**: 1543–1550.

Lürling, M. and Van Donk, E. 2000. Grazer-induced colony formation in *Scenedesmus*: are there costs to being colonial? *Oikos*, **88**: 111–118.

Lürling, M. and Verschoor, A.M. 2003. FO-spectra of chlorophyll fluorescence for the determination of zooplankton grazing. *Hydrobiologia*, **491**: 145–157.

Maynard Smith, J. and Szathmary, E. 1995. *The Major Transitions in Evolution*. Oxford: W.H. Freeman Spektrum.

Nakajima, T., Sano, A. and Matsuoka, H. 2009. Auto-/heterotrophic endosymbiosis evolves in a mature stage of ecosystem development in a microcosm composed of an alga, a bacterium and a ciliate. *BioSystems*, **96**: 127–135.

Nakajima, T., Matsubara, T., Ohta, Y. and Miyake, D. 2013. Exploitation or cooperation? Evolution of a host (ciliate)-benefiting alga in a long-term experimental microcosm culture. *BioSystems*, **113**: 127–139.

Nichols, S.A., Dayel, M.J. and King, N. 2009. Genomic, phylogenetic, and cell biological insights into metazoan origins. In *Animal Evolution: Genomes, Fossils and Trees* (M.J. Telford and D. Littlewood, eds.), pp. 24–32. Oxford: Oxford University Press.

Porter, K.G. 1976. Enhancement of algal growth and productivity by grazing zooplankton. *Science*, **192**: 1332–1334.

Raven, J. 1998. Book review: David L. Kirk. Volvox: Molecular-Genetic Origins of Multicellularity and Cellular Differentiation. Developmental and Cell Biology Series. Cambridge: Cambridge University Press. *Eur. J. Phycol.*, **33**: 275–280.

R Development Core Team. 2015. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Roper, M., Dayel, M.J., Pepper, R.E. and Koehl, M.A.R. 2013. Cooperatively generated stresslet flows supply fresh fluid to multicellular choanoflagellate colonies. *Phys. Rev. Lett.*, **110**: 1–5.

Ryther, J.H. 1954. Inhibitory effects of phytoplankton upon the feeding of *Daphnia magna* with reference to growth, reproduction, and survival. *Ecology*, **35**: 522–533.

Smith, J., Queller, D.C. and Strassmann, J.E. 2014. Fruiting bodies of the social amoeba *Dictyostelium discoideum* increase spore transport by *Drosophila*. *BMC Evol. Biol.*, **14**: 105.

Stanley, S.M. 1973. An ecological theory for the sudden origin of multicellular life in the late Precambrian. *Proc. Natl. Acad. Sci. USA*, **70**: 1486–1489.

Szathmáry, E. and Wolpert, L. 2003. The transition from single cells to multicellularity. In *Genetic and Cultural Evolution of Cooperation* (P. Hammerstein, ed.), pp. 271–290. Cambridge, MA: MIT Press.

Uchida, H., Yasumoto, K., Nishigami, A., Zweigenbaum, J.A., Kusumi, T. and Ooi, T. 2008. Time-of-flight LC/MS identification and confirmation of a kairomone in *Daphnia magna* cultured medium. *Bull. Chem. Soc. Jpn*, **81**: 298–300.

Van Donk, E., Lürling, M. and Lampert, W. 1999. Consumer-induced changes in phytoplankton: inducibility, costs, benefits and the impact on grazers. In *The Ecology and Evolution of Inducible Defenses* (R. Tollrian and C.D. Harvell, eds.), pp. 89–103. Princeton, NJ: Princeton University Press.

Velicer, G.J. and Yuen-tsu, N.Y. 2003. Evolution of novel cooperative swarming in the bacterium *Myxococcus xanthus*. *Nature*, **425**: 75–78.

Von Elert, E. and Franck, A. 1999. Colony formation in *Scenedesmus*: grazer-mediated release and chemical features of the infochemical. *J. Plankton Res.*, **21**: 789–804.

West, S.A., Fisher, R.M., Gardner, A. and Kiers, E.T. 2015. Major evolutionary transitions in individuality. *Proc. Natl. Acad. Sci. USA*, **112**: 10112–10119.

Yasumoto, K., Nishigami, A., Yasumoto, M., Kasai, F., Okada, Y., Kusumi, T. *et al.* 2005. Aliphatic sulfates released from *Daphnia* induce morphological defense of phytoplankton: isolation and synthesis of kairomones. *Tetrahedron Lett.*, **46**: 4765–4767.

Evolutionary Ecology Research — Volume 17 (2016)

Predation and the formation of multicellular groups in algae

APPENDIX

Stefania E. Kapsetaki *, Roberta M. Fisher & Stuart A. West

Department of Zoology, University of Oxford, South Parks Road,

The Tinbergen Building, Oxford, OX1 3PS, UK

* Corresponding author: stefania.kapsetaki@zoo.ox.ac.uk

REVISED 27-vii-2016

APPENDIX

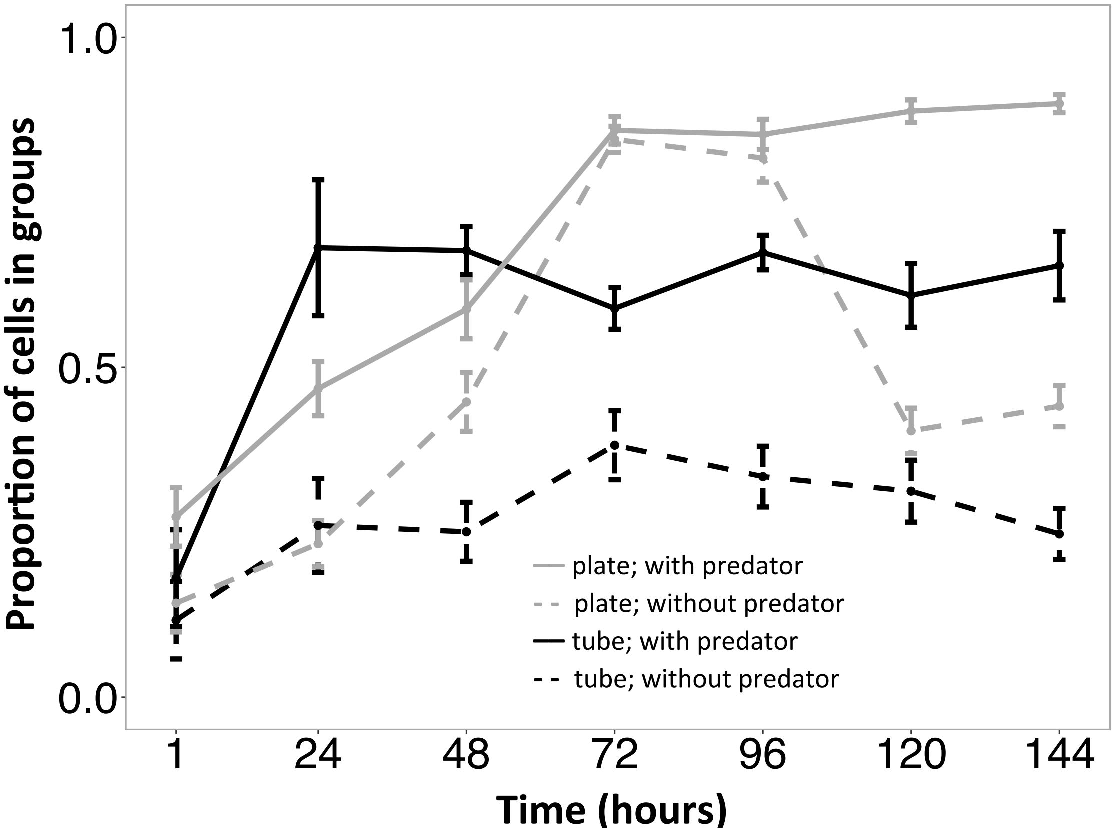


Figure S1. Comparison of the proportion of cells in groups between 50-mL falcon tubes and 24-well plates. In order to decide whether the 50-mL falcon tubes or the 24-well plate was the most suitable system to measure the proportion of cells in groups, we conducted the following experiment. We used nine 50-mL falcon tubes for the control and nine for the treatment, as well as nine wells on a 24-well plate for the control and nine for the treatment. In each tube we added 19.6 mL of *C. vulgaris* (1×10^5 cells mL^{-1}) to either 0.4 mL of PPY in the control or 0.4 mL of *Ochromonas* spp. (6×10^5 cells mL^{-1}) in the treatment set. We used the same cultures of *C. vulgaris* and *Ochromonas* spp. for the 24-well plate experiment. We added 980 μL of *C. vulgaris* in each well and 20 μL of PPY and *Ochromonas* spp. in the control and treatment, respectively. We incubated the falcon tubes and the 24-well plate at 20^0C light-dark 16:8 hour cycle and kept the tube caps loose to allow for oxygenation. We collected samples at seven time points after adding the putative predator: 1, 24, 48, 72, 96, 120, and 144 hours. At each time point, we tilted the falcon tubes five times, to homogenize the solution, and transferred 200 *μ*L of each culture into a 96-well plate. We acquired microscopy images of these subsamples and of the 24-well plate using a VisiCam digital camera under an inverted microscope (VWR International, Model XDS-3) at 20x magnification. We then quantified the proportion of cells in groups. In both cases the proportion of cells in groups was higher in the presence of the predator (tubes: glmmPQL, $P < 0.0001$; plates: glmmPQL, $P < 0.0001$). In the 24-well plate, in the absence of *Ochromonas* spp., 85% of cells were in groups within 72 hours. For this reason we chose to conduct our experiments using 50-mL falcon tubes. Error bars represent standard error of the mean (± se).

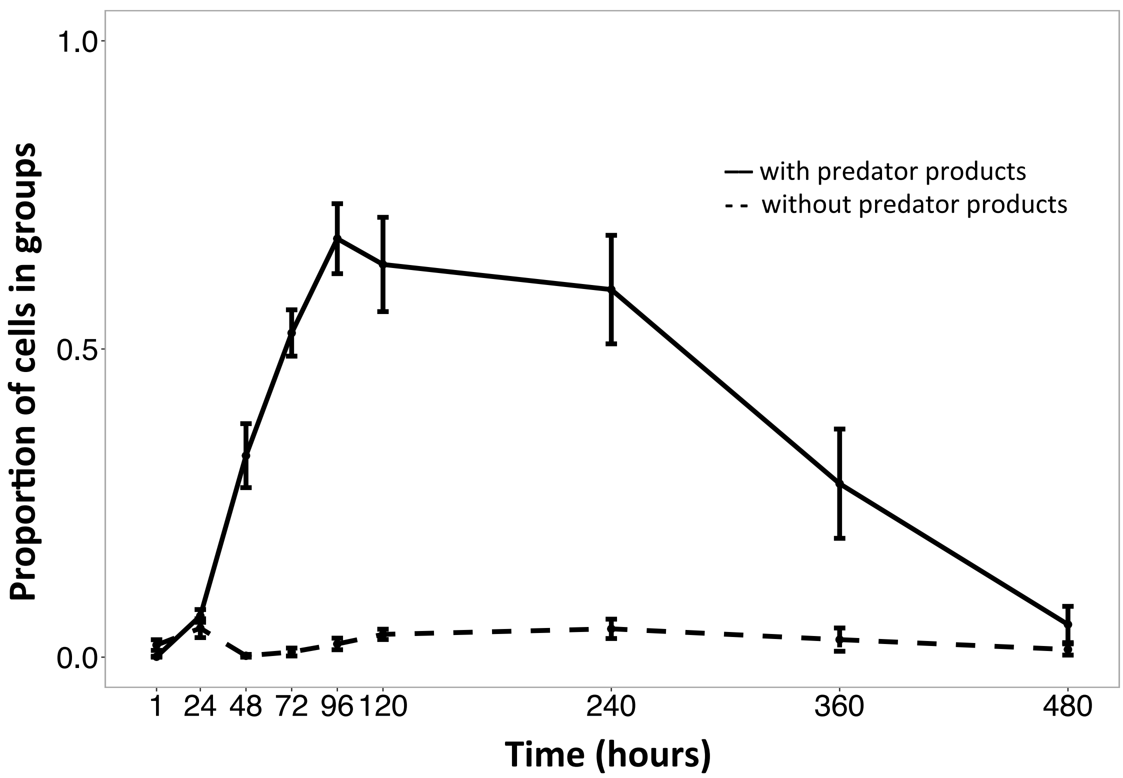


Figure S2. Proportion of *C. sorokiniana* cells in groups over 480 hours in the presence (solid line) and absence (dashed line) of *Daphnia* products. This Figure is an extension of Figure 6a.G. In the beginning of the experiment, the proportion of cells in groups did not differ significantly between the two treatments (glm at ‘time point 0 hours’, $F = 1.84$, $P = 0.192$). After 48 hours, the proportion of cells in groups increased in the presence of the *Daphnia* products (glm at ‘time point 48 hours’, $F = 68.12$, $P < 0.0001$), and the cells became again unicellular after 480 hours (glm at ‘time point 480 hours’, $F = 2.31$, $P = 0.147$). Error bars represent standard error of the mean (± se).

Evolutionary Ecology Research, 2017, **18**: 663–675

How do algae form multicellular groups?

Stefania E. Kapsetaki, Alexander Tep and Stuart A. West

Department of Zoology, University of Oxford, Oxford, UK

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.

Organisms: The freshwater algae *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus*, and the freshwater crustacean predator *Daphnia magna.*

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,

Correspondence: S.E. Kapsetaki, Department of Zoology, University of Oxford, South Parks Road, The Tinbergen Building, Oxford OX1 3PS, UK. email: stefania.kapsetaki@zoo.ox.ac.uk

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 Bolds Basal media at 20°C under a light/dark cycle of 16 : 8 hours using fluorescent illumination. We added 500 $\mu g \cdot mL^{-1}$ 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 Bolds 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* (10^6 cells $\cdot mL^{-1}$) 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 Bolds 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 10^6 cells $\cdot$ 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 Bolds 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 ($9 \times 3 = 27$ 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).

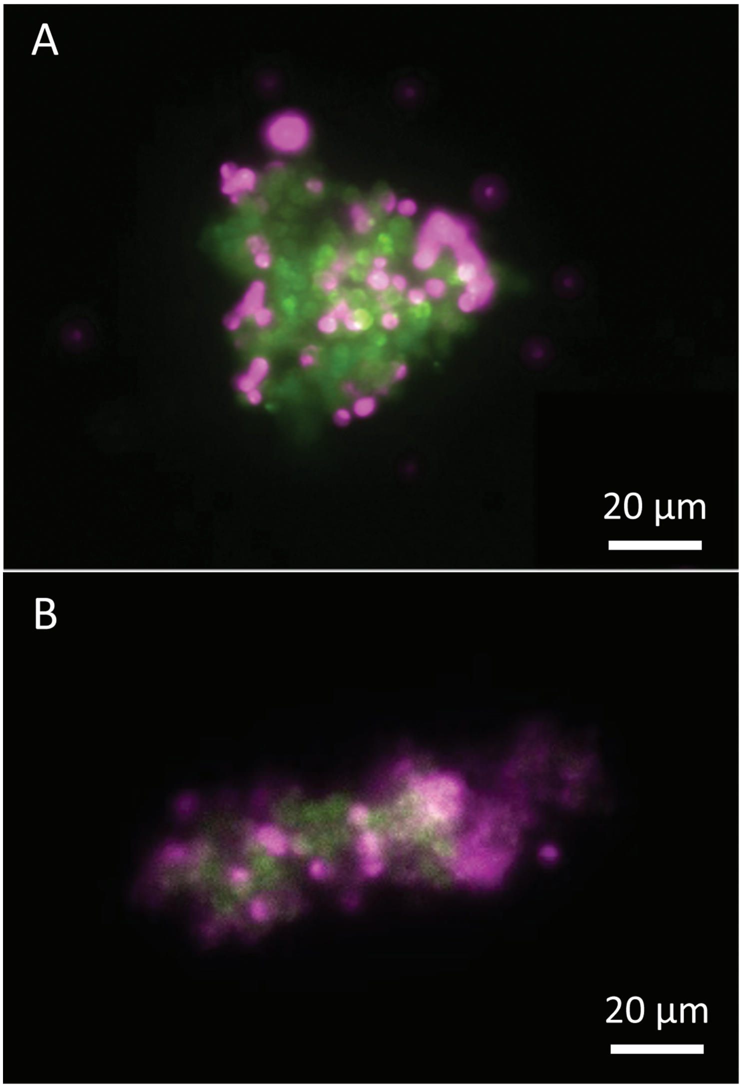


Fig. 1. Representative images of dichromatic groups within (A) and between (B) species. (A) Green- and violet-dyed *Chlorella sorokiniana* form a dichromatic group in the presence of *Daphnia*. (B) Green-dyed *Chlorella sorokiniana* and violet-dyed *Chlorella vulgaris* form a mixed-species dichromatic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

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.

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 Bolds Basal media to a final concentration of 4×10^5 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 ≥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 α is group size and p is the expected proportion of green cells. Specifically, p is $(b + r\alpha)/\alpha$, 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).

RESULTS

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 ± 20.6 hours (mean $\pm$ SEM), 10.8 ± 2.6 hours, and 51.6 ± 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*, $F = 11.10$,

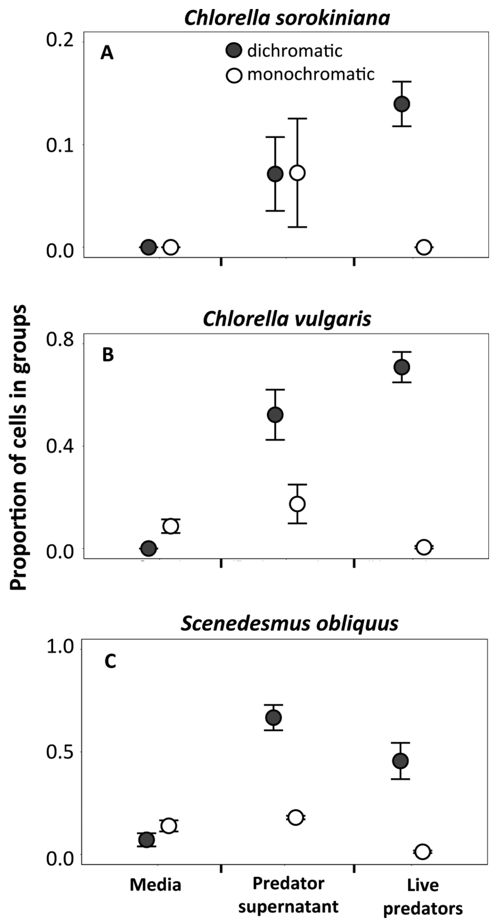


Fig. 2. Within-species group formation. The proportion of cells in groups is plotted in the absence of predators (media), the presence of predator supernatant, and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), 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.

$P = 0.009$, $df = 6$; *C. sorokiniana* with *S. obliquus*, $F = 15.19$, $P = 0.004$, $df = 6$; *C. vulgaris* with *S. obliquus*, $F = 36.90$, $P < 0.001$, $df = 6$; glm, media vs. predator supernatant and live predators: *C. sorokiniana* with *C. vulgaris*, $F = 23.88$, $P = 0.001$, $df = 7$; *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).

Furthermore, in all three algal species combinations the distribution of green cells in groups showed significantly more than binomial variation when exposed to predator

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

Algae	Predators	V_o/V_e	Number of groups sampled	χ^2-value	*P*-value
Within species					
Chlorella sorokiniana	supernatant	4.81	4	9.62	0.008
	live	1.63	3	1.63	0.201
Chlorella vulgaris	supernatant	7.49	36	254.71	<0.001
	live	68.20	6	272.81	<0.001
Scenedesmus obliquus	supernatant	23.75	221	5201.83	<0.001
	live	55.57	54	2889.77	<0.001
Between species					
C. sorokiniana and *C. vulgaris*	supernatant	6.22	32	186.78	<0.001
	live	17.90	12	179.03	<0.001
C. sorokiniana and *S. obliquus*	supernatant	17.48	24	384.61	<0.001
	live	24.00	28	624.07	<0.001
C. vulgaris and *S. obliquus*	supernatant	66.78	163	10752.68	<0.001
	live	39.29	58	2200.58	<0.001

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.

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).

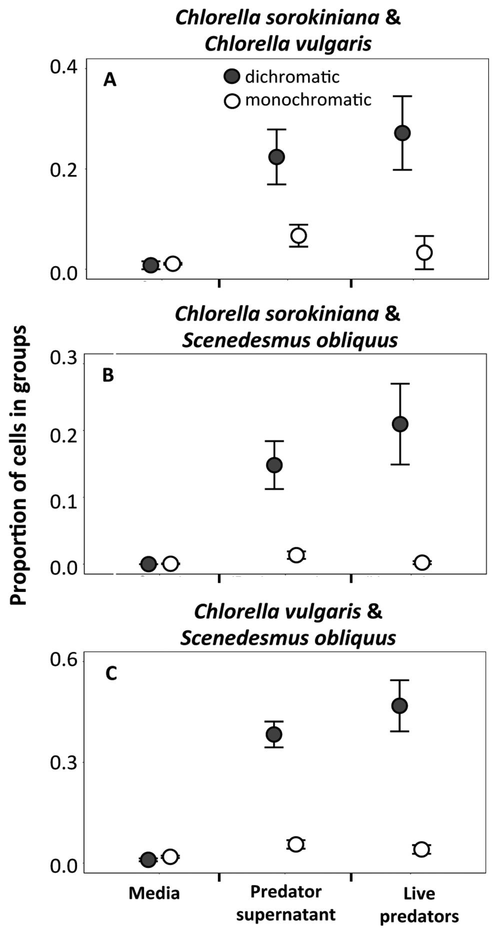


Fig. 3. Between-species group formation. The proportion of cells in groups is shown in the absence of predators (media), the presence of predator supernatant, and the presence of live predators. The cells are divided between those in groups containing only violet- or green-dyed cells (monochromatic), 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.

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

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. 3099Appendix – 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 (3099Appendix – 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 (3099Appendix – 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 (Kaushik *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 (Lurling 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.*, 2013). 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).

ACKNOWLEDGEMENTS

We thank Roni McGowan, Lindsay Turnbull, and Nick Hardberd for providing temporary lab space, Kevin Foster for allowing us to use the fluorescent stereoscope, Isabel Frost and Jamie Wheeler for valuable assistance in using the fluorescent stereoscope, and Roberta Fisher and David Crosse for helpful comments on the manuscript. This research was funded by the European Research Council (to S.A.W.), the HSBC (to A.T.), the Alexander S. Onassis Public Benefit Foundation, and the A.G. Leventis Foundation (to S.E.K.).

REFERENCES

Bonner, J.T. 1998. The origins of multicellularity. *Integr. Biol. Issues, News, Rev.*, **1**: 27–36.

Bonner, J.T. 2003. Evolution of development in the cellular slime molds. *Evol. Dev.*, **5**: 305–313.

Bonner, J.T. 2009. *The Social Amoebae: The Biology of Cellular Slime Molds*. Princeton, NJ: Princeton University Press.

Boomsma, J.J. 2007. Kin selection versus sexual selection: why the ends do not meet. *Curr. Biol.*, **17**: R673–R683.

Boomsma, J.J. 2009. Lifetime monogamy and the evolution of eusociality. *Phil. Trans. R. Soc. Lond. B: Biol. Sci.*, **364**: 3191–3207.

Boraas, M.E., Seale, D.B. and Boxhorn, J.E. 1998. Phagotrophy by a flagellate selects for colonial prey: a possible origin of multicellularity. *Evol. Ecol.*, **12**: 153–164.

Bourke, A.F.G. 2011. *Principles of Social Evolution*. Oxford: Oxford University Press.

Chavez-Dozal, A., Gorman, C., Erken, M., Steinberg, P.D., McDougald, D. and Nishiguchi, M.K. 2013. Predation response of *Vibrio fischeri* biofilms to bacterivorus protists. *Appl. Environ. Microbiol.*, **79**: 553–558.

Corno, G. and Jürgens, K. 2006. Direct and indirect effects of protist predation on population size structure of a bacterial strain with high phenotypic plasticity. *Appl. Environ. Microbiol.*, **72**: 78–86.

Fisher, R.M., Cornwallis, C.K. and West, S.A. 2013. Group formation, relatedness, and the evolution of multicellularity. *Curr. Biol.*, **23**: 1120–1125.

Fisher, R.M., Bell, T. and West, S.A. 2016. Multicellular group formation in response to predators in the alga *Chlorella vulgaris*. *J. Evol. Biol.*, **29**: 551–559.

Friman, V.P., Diggle, S.P. and Buckling, A. 2013. Protist predation can favour cooperation within bacterial species. *Biol. Lett.*, **9** (5): 20130548.

Green, R.F., Gordh, G. and Hawkins, B.A. 1982. Precise sex ratios in highly inbred parasitic wasps. *Am. Nat.*, **120**: 653–665.

Grosberg, R.K. and Strathmann, R.R. 1998. One cell, two cell, red cell, blue cell: the persistence of a unicellular stage in multicellular life histories. *Trends Ecol. Evol.*, **13**: 112–116.

Grosberg, R.K. and Strathmann, R.R. 2007. The evolution of multicellularity: a minor major transition? *Annu. Rev. Ecol. Evol. Syst.*, **38**: 621–654.

Gutzeit, G., Lorch, D., Weber, A., Engels, M. and Neis, U. 2005. Bioflocculent algal–bacterial biomass improves low-cost wastewater treatment. *Water Sci. Technol.*, **52**: 9–18.

Hamilton, W.D. 1964. The genetical evolution of social behaviour. I. *J. Theor. Biol.*, **7**: 1–16.

Hansen, S.K., Haagensen, J.A.J., Gjermansen, M., Jørgensen, T.M., Tolker-Nielsen, T. and Molin, S. 2007. Characterization of a *Pseudomonas putida* rough variant evolved in a mixed-species biofilm with *Acinetobacter* sp. strain C6. *J. Bacteriol.*, **189**: 4932–4943.

Harris, E.H. 2009. *The Chlamydomonas Sourcebook: Introduction to Chlamydomonas and its Laboratory Use*. San Diego, CA: Academic Press.

Kapsetaki, S.E., Fisher, R.M. and West, S.A. 2016. Predation and the formation of multicellular groups in algae. *Evol. Ecol. Res.*, **17**: 651–669.

Kaushik, S., Katoch, B. and Nanjundiah, V. 2006. Social behaviour in genetically heterogeneous groups of *Dictyostelium giganteum*. *Behav. Ecol. Sociobiol.*, **59**: 521–530.

Kirk, D.L. 2005. A twelve-step program for evolving multicellularity and a division of labor. *Bioessays*, **27**: 299–310.

Lampert, W., Rothhaupt, K.O. and von Elert, E. 1994. Chemical induction of colony formation in a green alga (*Scenedesmus acutus*) by grazers (*Daphnia*). *Limnol. Oceanogr.*, **39**: 1543–1550.

Lee, J., Cho, D.-H., Ramanan, R., Kim, B.-H., Oh, H.-M. and Kim, H.-S. 2013. Microalgae-associated bacteria play a key role in the flocculation of *Chlorella vulgaris*. *Bioresour. Technol.*, **131**: 195–201.

Lürling, M. 2001. Grazing-associated infochemicals induce colony formation in the green alga *Scenedesmus*. *Protist*, **152**: 7–16.

Lurling, M. and Beekman, W. 2006. Palmelloids formation in *Chlamydomonas reinhardtii*: defence against rotifer predators? *Ann. Limnol. – Int. J. Limnol.*, **42**: 65–72.

Lurling, M. and Van Donk, E. 2000. Grazer-induced colony formation in *Scenedesmus*: are there costs to being colonial? *Oikos*, **88**: 111–118.

Matz, C. and Kjelleberg, S. 2005. Off the hook – how bacteria survive protozoan grazing. *Trends Microbiol.*, **13**: 302–307.

Maynard Smith, J. and Szathmary, E. 1995. *The Major transitions in Evolution*. Oxford: W.H. Freeman Spektrum.

Mehdiabadi, N.J., Jack, C.N., Farnham, T.T., Platt, T.G., Kalla, S.E., Shaulsky, G. *et al.* 2006. Social evolution: kin preference in a social microbe. *Nature*, **442**: 881–882.

Michener, C.D. 1969. Comparative social behavior of bees. *Annu. Rev. Entomol.*, **14**: 299–342.

Michod, R.E. 2007. Evolution of individuality during the transition from unicellular to multicellular life. *Proc. Natl. Acad. Sci. USA*, **104**: 8613–8618.

Monier, J.-M. and Lindow, S.E. 2005. Spatial organization of dual-species bacterial aggregates on leaf surfaces. *Appl. Environ. Microbiol.*, **71**: 5484–5493.

Nilshammar, M. and Walles, B. 1974. Electron microscope studies on cell differentiation in synchronized cultures of the green alga *Scenedesmus*. *Protoplasma*, **79**: 317–332.

Quijano, G., Arcila, J.S. and Buitrón, G. 2017. Microalgal-bacterial aggregates: applications and perspectives for wastewater treatment. *Biotechnol. Adv.*, **35**: 772–781.

Raven, J. 1998. Book review: David L. Kirk. *Volvox: Molecular-Genetic Origins of Multicellularity and Cellular Differentiation*. Developmental and Cell Biology Series, editors J.D.L. Bard, P.W. Barlow, P.B. Green and D.L. Kirk. Cambridge University Press, Cambridge, 1998, xvi + 381 pp. *Eur. J. Phycol.*, 33: 275–280.

R Development Core Team. 2017. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Sathe, S. and Durand, P.M. 2016. Cellular aggregation in *Chlamydomonas* (Chlorophyceae) is chimaeric and depends on traits like cell size and motility. *Eur. J. Phycol.*, **51**: 129–138.

Sathe, S., Kaushik, S., Lalremruata, A., Aggarwal, R.K., Cavender, J.C. and Nanjundiah, V. 2010. Genetic heterogeneity in wild isolates of cellular slime mold social groups. *Microb. Ecol.*, **60**: 137–148.

Sathe, S., Khetan, N. and Nanjundiah, V. 2014. Interspecies and intraspecies interactions in social amoebae. *J. Evol. Biol.*, **27**: 349–362.

Solari, C.A., Galzenati, V.J. and Kessler, J.O. 2015. The evolutionary ecology of multicellularity: the volvocine green algae as a case study. In *Evolutionary Transitions to Multicellular Life: Principles and Mechanisms* (I. Ruiz-Trillo and A.M. Nedelcu, eds.), pp. 201–223. Dordrecht: Springer.

Trainor, F.R. 1998. *Biological Aspects of Scenedesmus (Chlorophyceae) – Phenotypic Plasticity*. Stuttgart: J. Cramer.

Trainor, F.R., Cain, J.R. and Shubert, L.E. 1976. Morphology and nutrition of the colonial green alga *Scenedesmus*: 80 years later. *Bot. Rev.*, **42**: 5–25.

Velicer, G.J. and Vos, M. 2009. Sociobiology of the myxobacteria. *Annu. Rev. Microbiol.*, **63**: 599–623.

West, S.A., Fisher, R.M., Gardner, A. and Kiers, E.T. 2015. Major evolutionary transitions in individuality. *Proc. Natl. Acad. Sci. USA*, **112**: 1–8.

Yamamoto, M., Kurihara, I. and Kawano, S. 2005. Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae). *Planta*, **221**: 766–775.