The ecology and evolution of multicellularity in green algae

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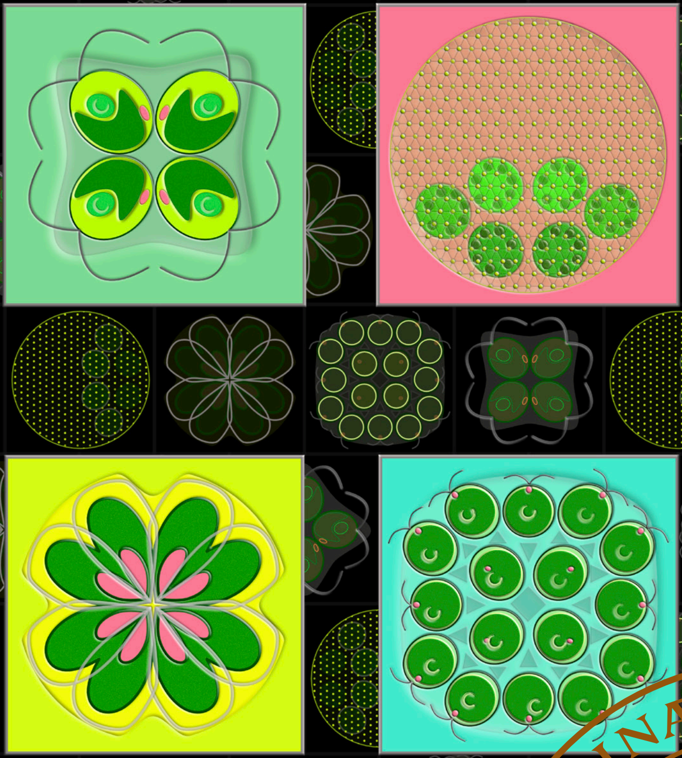

To mom / 致亲爱的妈妈

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Qinyang Li

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Key words: Multicellularity, ecology, stress, reactive oxygen species, physiology, green algae, Chlorophyta, plasticity, growth rates, trade-offs, stoichiometry, nitrogen, phosphorus, freshwater, niche, species distribution model, life history, predation

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“When men work in pairs, one sees advantages the other would miss, while a man on his own may see the possibilities, but lack the necessary imagination and intelligence."

– Diomedes knows the value of cooperation, in *The Iliad.*

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Abstract

The evolution of multicellularity is one of the major evolutionary transitions that has shaped the diversity of life on Earth. Multicellular organisms have arisen numerous times across the tree of life, but explaining why some lineages are multicellular while others are unicellular remains puzzling. In my thesis, we tested how the ecological variables may differentially favour multicellular and unicellular green algae (N=82). Specifically, we examined the fitness consequences of being unicellular, facultatively multicellularity and obligately multicellular across fluctuating environments (Paper I, II, III). We identified the physiological responses that were associated with facultative and obligate multicellularity (Paper II, III). We also modelled the ecological differentiation between unicellular and multicellular species in nature (Paper I, III, IV). We found that multicellularity in unicellular green algae is inducible by a range of environmental disturbances including predation, turbulence, nutrient imbalance and temperature variations. Importantly, we show that it is more of the severity of environmental stress rather than the type of the environment that triggers multicellularity in green algae. We further establish the link between multicellular group formation to unicellular stress response physiology, indicated by increased reactive oxygen species and starch contents. In Paper III, we found that multicellular algae have different life-history traits than unicellular algae, typically growing slower but have better survival over deteriorating environment. Such difference may have been one key cause to the niche differentiation between multicellular and unicellular green algae in the wild, shown in Paper IV.

Popular science summary

Through the history of life, unicellular organisms in some lineages abandoned their solitude and embraced to live in an interdependent collective. This simple "lifestyle" change, creating multicellular organisms like plants, fungi and animals, has fundamentally altered our Earth's biosphere.

However, compared to an animal or a plant, a single cell is vastly more efficient at replicating its own genetic material, the ultimate evolutionary currency. This makes the evolution of multicellularity quite counterintuitive – why choosing such a slow path?

A cell needs nutrients, water, stable temperature and many other factors to stay alive. But environmental elements can be harsh, posing significant pressure on solitary cells. By grouping together, cells can better buffer such environmental variations. Additionally, grouping together increases size by default, making the collective more resistant to predation and can helping them explore new environmental opportunities. If grouping increases a cell's chance to survive and reproduce, such grouping behaviour will be favoured over time, giving rise to multicellularity.

In this thesis, we test the role of environmental stressors in multicellularity using freshwater green algae. By subjecting many different algae in different conditions, such as presence of predation and turbulence, changing temperature, nutrients and salt concentrations. We also compiled all the freshwater green algae found in Sweden and compared the distribution dynamics between in total 114 unicellular and multicellular genera.

We find that most green algae can be induced to form groups when conditions get bad. And this is linked to algal cell's physiological response to stress. In adverse conditions, the oxidative stress level rises in algae and leads to increased production of sticky metabolic products such as starch and sugar-rich mucus layer around the cell – likely making cells more prone to stick to each other. The benefits of unicellular algae being stuck together wasn't obvious as we found that cells within such groups reproduce slower than when they are alone. But by studying multicellular algae, we found that, though they grow slower indeed, they survive for longer in a deteriorating environment. This insight points to different life-history strategy between unicellular and multicellular algae - the former have a boom-and-bust way of life while the latter is more resilient against environmental stress. This difference in strategy may also explain the occurrence pattern between unicellular and multicellular algae in natural lakes – multicellular algae increase much more than unicellular algae during summertime, when nutrients are low, and predation is high.

What we learned in the end, is that cells do suffer a reproductive cost in a multicellular group. But group living have its perks, too. If a cell has the tendency to form groups, like the sticky-compound-producing algae, and if the environment keeps promoting their grouping. A previously solitary cell may well be on its way to becoming a vital part of a thriving collective.

科普摘要

在生命的演化历程中，一些单细胞生物放弃了独立自主，开始过上相互依赖的群体生活。这种看似简单的“生活方式”改变，催生出了植物、真菌和动物等多细胞生物，彻底改变了地球的生物圈。

然而，相比于动物或植物，单细胞生物在复制自身遗传物质——这种“进化货币”——方面效率极高。因此，多细胞生物的演化看起来并不直观：为何要选择一条更慢的发展道路？

一个细胞需要营养、水分、稳定的温度等诸多因素才能生存。而环境往往变化无常，给独立生活的细胞带来了很大压力。通过聚集成群，细胞能够更好地缓冲这些环境波动。此外，群体的体积更大，从而更能抵御捕食者的攻击，并有能力探索新的生态机会。如果结群能提高细胞的生存与繁殖机会，那么这种行为就会在进化中被自然选择，从而促成多细胞生物的诞生。

在本论文中，我们利用淡水绿藻研究了环境压力在多细胞演化中的作用。我们将多种不同的绿藻暴露于不同的实验条件下，例如捕食者的存在、温度变化、营养和盐度水平等。同时，我们还调查了瑞典所有淡水绿藻的种类，并比较了其中 114 个单细胞与多细胞属的分布动态。

我们发现，大多数绿藻在恶劣条件下可以被诱导形成群体。这种现象与细胞对压力的生理反应密切相关：在不利环境中，绿藻的氧化应激水平升高，促使其产生更多黏性代谢产物，例如淀粉和富含糖分的黏液层，包裹在细胞周围，使细胞更容易彼此粘附在一起。然而，单细胞绿藻聚集的好处并不明显——我们发现它们在群体中繁殖得比单独时更慢。但在研究多细胞藻类时，我们发现它们虽然生长速度较慢，却能在恶化的环境中存活得更久。这一发现表明单细胞与多细胞藻类采取了不同的生活史策略：前者“繁荣-崩溃”起伏大，而后者则更具抗压能力。这种策略差异也可能解释了它们在自然湖泊中的分布模式——夏季时，营养匮乏、捕食压力上升，多细胞藻类的数量增长远超过单细胞藻类。

最终，我们认识到：加入多细胞群体的确会让细胞付出繁殖上的代价。但群体生活也有其独特的优势。如果一个细胞本身具有结群倾向（例如产生黏性物质的藻类），并且环境持续推动这种群体形成，那么原本独居的细胞就可能踏上成为一个繁荣群体关键一员的演化之路。

Introduction

Multicellularity as one of the major transitions in evolution

Life originated ~3.7 billion years ago, creating a new realm where biological entities roam (Javaux, 2019; Rosing, 1999). These entities evolved to be more complex over time, from simple replicating molecules to multicellular organisms comprised of trillions of differentiated cells. The common feature shared among these changes is that individuals lose their autonomy to reproduce by themselves and become parts of a cooperative group emerged as a higher unit of selection (Gardner & Grafen, 2009). This phenomenon, including the evolution of genomes, eukaryotic cells, multicellular organisms and eusocial society, is collectively termed a major evolutionary transition (Buss, 1987; Smith & Szathmáry, 1995; Szathmáry, 2015; West et al., 2015).

Major evolutionary transitions are products of cooperation (Box 1). There are two main explanations for how cooperation shapes major evolutionary transitions. The first explanation is that genetically distinct parties gain benefits by complementing each other in their functional roles, a phenomenon termed mutual benefit (Andersson et al., 2022; Gardner & Foster, 2008; West et al., 2007). For example, a eukaryotic cell is a chimera of organelles performing different functions, some of them have evolved from bacteria , such as ATP-producing mitochondria and photosynthetic chloroplasts (John & Whatley, 1975; W. F. Martin et al., 2015; W. Martin & Kowallik, 1999; Palmer, 2003; Sagan, 1967); Similarly, in symbiotic organisms, partnerships are formed to synergise and exchange key resources, typified by lichens made by the association among fungi, algae (or cyanobacteria) and yeasts. (Andersson et al., 2022; Bourrat & Griffiths, 2018; Spribille et al., 2016). Such major evolutionary transitions, formed by partners with different genetic makeups, have been referred to as ‘egalitarian’ (D. C. Queller, 1997, 2000).

The second explanation is that genetically similar parties cooperate, with actors increasing the fitness of related recipients at a cost to themselves, a phenomenon known as altruism (Hamilton, 1964; West & Gardner, 2010). For instance, castes in ants, termites, bees, and even naked mole rates forgo their own reproduction to promote the fertility of their queens and kings (Boomsma et al., 2011; Hughes et al., 2008; Ross & Keller, 1995). Somatic cells of multicellular organisms are analogous to sterile castes in a eusocial colony, maintaining and protecting reproductive germ cells, the cellular equivalent to queens and kings (Bonner, 1993; Boomsma & Gawne, 2018). Major evolutionary transitions involving genetically related individuals have been referred to as ‘fraternal’ (D. C. Queller, 1997, 2000). Here I focus on the evolution of multicellularity, one of the fraternal major evolutionary transitions that has arose numerous times during the history of the earth, and that has dramatically transformed the natural world (Lamża, 2023; J. Umen & Herron, 2021).

Box 1 – Social interactions and cooperation

Mutual benefit and altruism, together with selfishness and spite, are the four principal interactions between biological entities (Hamilton, 1964). They are defined in term of fitness outcomes of the parties involved: the actor and the recipient. Mutual benefit brings mutual fitness gains. Selfishness happens when an actor exploits a recipient to their detriment. Altruism is the opposite of selfishness and spite leads to fitness loss in both parties (Table 1).

Such interactions can happen at any biological level. For example, meiotic drive is a case of selfishness at the genome level, where one actor (a gene) actively biases the odds of transmission by manipulating meiosis, resulting in recipient genes having lower transmission probabilities (Buckler et al., 1999). Spiteful behaviours have been observed in bacterial warfare, where bacteriocin, an anti-microbial compound, were produced by bacteria themselves (Gardner et al., 2004; Riley & Wertz, 2002).

In multicellular organisms, somatic cells act altruistically towards germline cells, whereas cancer cells are examples of selfish cheats who proliferate indefinitely and exploit common recourses along the way (Aktipis et al., 2015; Brown et al., 2023; A. M. Nedelcu, 2020). It is easy to understand that selection favours fitness enhancing behaviours, such as selfishness and mutual benefit. To make sense of the fitness reducing behaviours like altruism and spite, however, is more challenging. This is where inclusive fitness theory can help.

Box 1 (cont.)

Inclusive fitness theory partitions an individual's total fitness ('inclusive') into direct and indirect fitness. Direct fitness refers to the reproductive success of an individual, whereas indirect fitness is the effect of an individual on the reproductive success of its relatives. Therefore, actions that harm an individual's direct fitness (altruism and spite) can be evolutionarily favoured if they provide a large enough indirect fitness benefit. This principle

Table 1 Social interactions defined by expected fitness change between an actor and a recipient**.**

		Expected fitness return of the recipient	
		Gains (+)	Loses (-)
Expected fitness return of the actor	Gains (+)	Mutual benefits	Selfishness
	Loses (-)	Altruism	Spite

After Bourke (2011) who was after Hamilton (1964)

Inclusive fitness theory partitions an individual's total fitness ('inclusive') into direct and indirect fitness. Direct fitness refers to the reproductive success of an individual, whereas indirect fitness is the effect of an individual on the reproductive success of its relatives. Therefore, actions that harm an individual's direct fitness (altruism and spite) can be evolutionarily favoured if they provide a large enough indirect fitness benefit. This principle

is captured by Hamilton's rule: from the actor's point of view, if the cost, c, (i.e., the reduction in direct fitness) of performing an action is compensated by the benefit, b, (i.e., the increase in the direct fitness of recipients) weighted by the relatedness to the recipient, r, genes for such actions will be selected for (Hamilton, 1964). This inequality known as Hamilton's rule, $\boldsymbol{rb - c > 0}$, lays out the foundation for an evolutionary understanding of social interactions.

The evolution of multicellularity

Multicellularity exemplifies biological interactions at the cellular level. Like all major evolutionary transitions, the evolution of multicellularity happens in three stages, group formation, group maintenance and group transformation (Bourke, 2011) (Fig 1). The initial formation of a group is a key event that determines levels of relatedness between cells ('Origin of groups') (D. C. Queller, 2000). Once groups form, group phenotypes are exposed to selection that determines whether multicellular groups are maintained over generations, or groups disassociate to resume a unicellular life. If multicellular groups are maintained, selection can favour adaptations, such as cell specialisation, that transform and increase the fitness of groups (West et al., 2015). I will discuss these three phases of the transition to multicellularity, examining what is known about the selective and mechanistic basis to each phase and what key questions remain to be addressed.

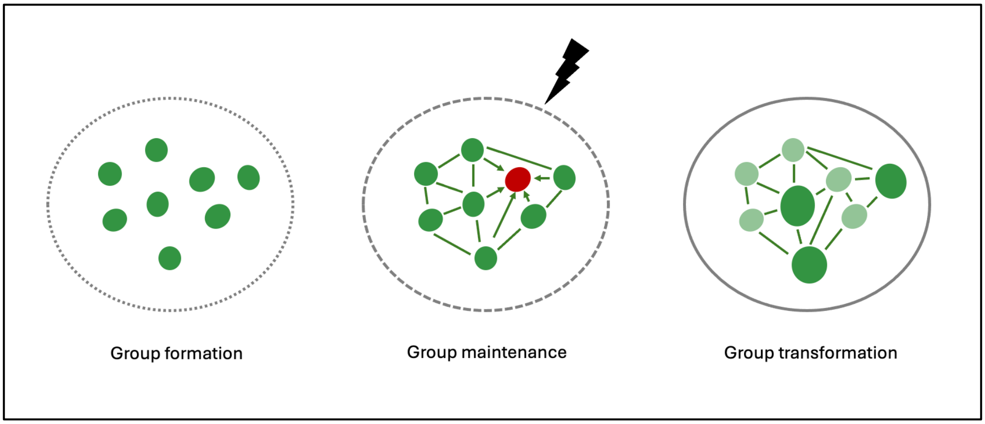

Figure 1. The path to multicellularity

The evolution of multicellularity can be viewed in three stages. Group formation, where cells forming groups through mechanisms such as cell adhesion or chemosensing. Group maintenance, where the stability of the group depends on managing selfish elements from within, and warding off stressors from the environment. Group transformation, where interdependence among cells evolve due to their increased specialisation.

The path to multicellularity – group formation

There are many ways to classify multicellularity, but one key distinction is how multicellular groups form (O'Malley, 2022). Groups can form either by different cells gathering ("Aggregative multicellularity"), or clonal cells remaining together after cell division ("Clonal multicellularity") (Herron, 2022; Niklas & Newman, 2016; Ruiz-Trillo & Nedelcu, 2015). Cells within a clonal group have a relatedness of 1, which aligns the fitness interests among clones. This appears a crucial condition favouring the evolution of specialised cell types where altruistic somatic cells seemingly perform various functional "services" to the germ cells (Fisher et al., 2013). In contrast, when multicellular groups form through aggregation there is the potential for relatedness among cells to be less than 1. For example, in the cellular slime mould *Dictyostelid discoideum*, resource limitation triggers individual amoebas to form an aggregative multicellular slug with increased foraging speed and nutrient transfer (Bonner, 2001). Slugs also form a fruiting body that consists of altruistic non-reproductive stalk cells that elevate reproductive spores to aid their aerial dispersal. This appears a common strategy as similar morphologies have been documented in taxa such as *Myxobacteria*, *Bacillus*, *Pseudomonas,* and some ciliates (*Sorogena*) (Bonner, 1997). However, if slugs contain genetically distinct individuals (i.e., $r < 1$), cheaters can bias their representation in the reproductive spores, exploiting the altruism by stalk forming cells. This "vulnerability" of aggregative multicellularity, is thought to limit the evolution of multicellular complexity, such as the number of cell types (Kessin, 2000).

Hamilton's rule (Box1) specifies the conditions where selection favours group-level adaptations, but it does not explain the origin of groups. Instead, molecular and cell biology research has revealed mechanisms such as adhesion, chemotaxis and kin recognition that can explain group formation. Clonal multicellularity requires forms of cell adhesion that prevents daughter cells from dispersing, whereas aggregate multicellularity often requires mechanisms such as chemotaxis that enable cells to find each other. For example, in metazoans, a type of transmembrane protein called cadherins enable cell-to-cell adhesion and integrins, another type of protein receptor, promotes cell-to-extra cellular matrix adhesion (Nichols et al., 2012). In plants and fungi, similar functions are accomplished using hemicelluloses and glycoproteins respectively (Abedin & King, 2010; Buss, 1987). Taxa with migrating cells, such as animals and cellular slime moulds, have independently evolved calcium-dependent homophilic adhesion molecules (Abedin & King, 2010; King et al., 2008; Nichols et al., 2012; Sebé-Pedrós et al., 2010; Suga et al., 2013).

Chemosensing behaviours and adhesion molecules may also facilitate genetic kin recognition, especially when groups form through aggregation. Genetic kin recognition can help stabilise the formation and maintenance of groups by increasing relatedness among cells, and the indirect fitness benefits of multicellularity. For example, the cellular slime mold, *Dictyostelium discoideum* uses chemosensing by secreting cyclic adenosine monophosphate (cAMP) to coordinates aggregation (Du et al., 2015; Firtel & Chapman, 1990). In the fruit body developmental phases of *D. discoideum*, proteins such as *csA* can preferentially benefit amoebas carrying the same gene, ensuring more *csA* carriers end up as reproductive spores (Abedin & King, 2010; Queller et al., 2003). Similarly, in baker's yeast, *Saccharomyces cerevisiae*, the FLO1 gene regulates the production of adhesive proteins for multicellular group formation, a process called flocculation. FLO1 expressing yeast cells also preferentially stick to other FLO1 carriers, and cells within flocs have higher resistance to environmental stressors, such as hydrogen peroxide, ethanol, and antibiotics (Kuzdzal-Fick et al., 2019; Smukalla et al., 2008).

The path to multicellularity – group maintenance

Once a group has formed, its maintenance depends on two factors: 1) whether the group phenotype confers a selective advantage, which depends on the environment, and 2) whether exploitation by selfish members ('cheats') can be controlled. To understand how the environment selects for multicellularity, it is helpful to examine unicellular versus multicellular organisms closely.

There are a number of key differences between unicellular and multicellular organisms. Firstly, multicellular groups result in cells experiencing one of two environments, external and internal (Gerhart & Kirschner, 2000). The internal environment buffers against various stressors (Bonner, 1997; Jezberová & Komárková, 2007), such as salinity (Khona et al., 2016), oxidation (Čáp et al., 2012; Hammarlund, 2020), and temperature (Duan et al., 2018). Secondly, multicellularity requires cell communication and coordination. Cells communicate through kinase signalling, and the expansion of various types of kinases have been reported to be important in the evolution of animals (Sebé-Pedrós et al., 2010), plants (Bowles et al., 2020), and brown algae (Cock et al., 2010). An efficient system for coordinating cell processes can result in significant group-level adaptation, but presumably in the early stages of multicellular evolution, cell coordination systems are rudimentary, generating barriers to multicellularity (Solari et al., 2015). Thirdly, multicellular organisms are generally larger than unicellular ones. The increased size, while proposed to mitigate predation pressure in many experiments (Fisher et al., 2016; Jezberová & Komárková, 2007; Lürling & Van Donk, 1997, 2000), reduces the relative surface area of an organism and hence nutrient uptake and metabolic efficiency (Bestová et al., 2021; Lynch, 2024; Lynch et al., 2022; A. Nedelcu & Michod, 2002; White & Marshall, 2023).

Even within multicellular organisms, dynamic ecological factors can further alter the selective advantages of aggregative versus clonal multicellularity. Clonal group formation requires multiple cell division cycles, but aggregative multicellularity requires no cell division at all. This is why short-term changes in the environment, such as desiccation risk and resource patchiness, can select for fast group formation brought about by aggregative multicellularity (Márquez-Zacarías et al., 2021). In support of this idea, aggregative multicellular organisms are typically facultative (i.e., individual cells retain their ability to replicate) (Márquez-Zacarías et al., 2021). They are also more likely to occur in terrestrial environments, with patchy resources and more severe environmental fluctuations than aquatic environments (Bonner, 1997; Fisher et al., 2020).

Surviving short-term environmental changes is no guarantee that multicellular phenotypes will persist over longer, evolutionary timescales. This requires controlling the exploitation of cells from within the multicellular group (selfish cheats). For example, aggregative multicellularity has the potential for relatedness among cells to be lower than 1, opening the door for less-related cells to exploit common goods and destabilise the collective. This was demonstrated in bacteria *Pseudomonas fluorescens*, where a mutant genotype forms multicellular biofilm by producing costly polymers (altruists). The integrity of such biofilms was shown to be vulnerable to the invasion of non-polymer-producing strains (cheats) (Rainey & Rainey, 2003; Spiers et al., 2002). Similarly, *S. cerevisiae* has mutant strains that creates either clonal multicellularity (ace2+) or aggregative multicellularity (FLO1+). Clonal strain were shown to be more competitive when co-cultured with aggregative strain (Pentz et al., 2020).

One key life-cycle trait thought to be crucial in controlling cheating is a single-celled bottleneck during development (Wolpert & Szathmáry, 2002). This ensures that all daughter cell lines are genetically identical being descended from the same mother cell, a hallmark of clonal multicellularity. This is why transformation to complex multicellularity, where large numbers of cells and altruistic cell types form a highly coordinated entity, are thought to only have evolved in clonal multicellular lineages (Fisher et al., 2013; Howe et al., 2024; Márquez-Zacarías et al., 2021; Pentz et al., 2023).

The path to multicellularity – group transformation

Social group transformation refers to when a group transcends from a collection of its members (cells) to a cohesive, higher-level entity (an individual) (Bourke, 2011; Okasha, 2008). This means members within a group evolve interdependence and cannot survive or reproduce alone. The evolution of interdependence is solidified when cells differentiate to perform specialised tasks, also known as a division of labour. Selection for the division of labour is expected to occur when there are accelerating fitness returns from cells performing specific functions. Alternatively, if there are additive or diminishing fitness returns from cells performing specific tasks then generalist strategies are predicted to evolve that may explain the presence of multicellular groups with undifferentiated cells (Michod, 2006, 2007; West & Cooper, 2016).

The most significant division of labour is between reproductive functions and survival functions (reproductive division of labour). This is because that reproduction and survival functions within a single cell directly trade-off with each other, while multicellular organisms with reproductive division of labour can decouple such a link and maximising both reproductive function by germ cells and survival function by somatic cells (Buss, 1987; Koufopanou, 1994; A. Nedelcu & Michod, 2002). One mechanistic driver for reproductive division of labour is that some organelles can have dual functions. For example, the basil bodies of flagella are used for locomotion when cell are not dividing (somatic function) but are required to form centrioles during mitosis (reproductive function). This is a fundamental constraint preventing cells from being motile while reproducing (Buss, 1987; Koufopanou, 1994; A. Nedelcu & Michod, 2002; Rueffler et al., 2012; Szathmáry & Smith, 1995). From an evolutionary perspective, if a reproductive division of labour increases the overall number of progenies a group produces (higher fitness), cells are expected to either maximize investment in reproductive traits (e.g., large cell size, no flagella) or investment in traits linked to somatic maintenance and survival (e.g., immune function, oxygen transportation, etc.) (West & Cooper, 2016). Once freed from the high energetic demands of reproduction, cells can reallocate energy towards new specialised cell types. Therefore, the evolution of a reproductive division of labour is shown to precede the differentiation of somatic cell types (Simpson, 2012).

Cell differentiation mechanisms are coordinated by the interactions between genetic programs and non-genetic cues (e.g., developmental programming initiated by environmental cues). In the green alga *Volvox carteri,* which consists of a few large immotile germ cells and some 2,000 small flagellated somatic cells, cell size differences after the initial 5 rounds of division act as the cue for germ-soma differentiation (Kirk, 1997). The slightly larger cells stop dividing, lose flagella and increase in size, whereas the smaller cells continue mitosis. This process is regulated by the *regA* gene (Hallmann, 2011; Kirk, 1997; A. M. Nedelcu, 2009; A. M. Nedelcu & Michod, 2006). Many cyanobacteria species have a specialised non-reproductive cell type called heterocysts for nitrogen fixation. Heterocysts appear at regular intervals along the filaments and their development is regulated by genes such as *hetC* and *asr1734* (Kumar et al., 2010). In the multicellular slug phase of *D. discoideum*, anterior cells experience higher concentrations of oxygen and differentiate into stalk cells. Such chemotaxis and subsequent morphogenesis are mediated by a family of chlorinated alkyl hexaphenones molecules called Differentiation-inducing factor (DIF) (Bonner, 2001; Kubohara et al., 2017).

Unresolved issues within the field of multicellularity

Despite our understanding of multicellularity advancing on multiple fronts, there remain many unanswered questions. One major gap in our knowledge is what ecological factors influence the formation, maintenance, and transformation of multicellular groups. This gap manifests itself in several open questions. Firstly, the early conditions favouring the origin of multicellularity are poorly known (Knoll, 2022; Simpson, 2012, 2023). The effect of predation has been championed as the main driver for multicellularity, yet predation is unlikely to have favoured the rise of the first multicellular organisms due to the limited size of single celled predatory organisms. In addition, predation can also reduce group size (Bergkvist et al., 2012; Selander et al., 2019), indicating that the relationship between predation and multicellularity depends on variation in the characteristics of predators. Secondly, we know that many genes performing vital functions in extent multicellular species were co-opted from unicellular ancestors (Grochau ‐ Wright et al., 2017; König & Nedelcu, 2020; A. M. Nedelcu & Michod, 2006). Yet the original functions of these co-opted genes remain largely unknown. Understanding the complexity of unicellular organism life cycles with and without closely related multicellular ancestors, may provide important insights into the processes that are co-opted during multicellular evolution. This has led to the hypothesis that multicellularity is in some part a transition from temporally differentiated cell states in unicellular organisms to a spatially differentiated cells in multicellular species (König & Nedelcu, 2020; Love & Wagner, 2022; Mikhailov et al., 2009; A. M. Nedelcu, 2009). We lack both the ecological context selecting for functions of such co-opted genes in unicellular ancestors, and the conditions that drive the shift from temporal to spatial differentiation. Thirdly, there are many examples of closely related unicellular and multicellular species that, coexist. What factors have maintained their morphological distinction under apparently similar environmental condition remains unknown.

A lot of these open questions can be addressed by gaining a greater understanding of the ecological conditions favouring multicellularity. The evolution of multicellularity and other major transitions are about the cost and benefits of cooperation and ecology plays a driving role in this calculation. For instance, one species of choanoflagellate, the closest unicellular relative to animals, has five life cycle stages ranging from sessile, slow & fast unicellular stages to chain- and rosette- forming multicellular stages (Dayel et al., 2011). If we could identify the environmental drivers behind such morphological diversity, we would potentially understand the ecological niches differentiating unicellular and multicellular life stages. Incorporating ecological variables, however, is challenging as they vary in multiple dimensions, but such variability is likely to be important in determining the strength of selection for and against multicellular life (Black et al., 2020).

Aims of the thesis

In this thesis, I examine the role of ecology in the evolution of multicellularity across green algae. Green algae have undergone multiple independent transitions to multicellularity (J. Umen & Herron, 2021) and can be studied in the laboratory and the wild. I combine comparative experiments in the lab and correlational studies relating the prevalence of freshwater unicellular and multicellular green algae to their natural environment, to examining the causal role of ecology in determining the costs and benefits of multicellularity. The overarching aim of my thesis is to address: ***What ecological niches are unicellular and multicellular green algae adapted to?***

Specifically, I:

1) Examine the fitness costs and benefits of being unicellular, facultatively multicellularity and obligately multicellular across fluctuating nutrient environments (Paper I, II, III).

2) Identify the physiological mechanisms that are associated with facultative and obligate multicellularity (Paper II, III).

3) Model the ecological divergence between unicellular and multicellular species in nature (Paper I, III, IV).

Study system

Multicellularity and volvocine algae

I will primarily study the planktonic chlorophyte algae, specifically focusing on the volvocine algae. They include three closely related families of freshwater multicellular species (Tetrabaenaceae, Goniaceae and Volvocaceae) and their unicellular relatives from the genera *Chlamydomonas*, *Vitreochlamys* and *Lobomonas* (J. Umen & Herron, 2021).

The range of multicellular complexity within the volvocine algae manifests in many ways including different cell numbers, cell types, and reproductive and developmental modes (Fig 2). The number of cells species have within volvocine algae ranges from single celled species, such as *Chlamydomonas*, to curved sheets of 4-64 undifferentiated cells, such as in *Tetrabaena* and *Gonium*, to the spherical bodies comprised of 8-50,000 cells in the cases of *Astrophomene*, *Pandorina*, *Eudorina*, *Pleodorina* and *Volvox*. During embryogenesis, multicellular volvocine algae need to expose their flagella outwards, a process called inversion. Different species achieve this goal differently. For example, *Eudorina* "peels" itself inside out to expose flagella before forming the spherical colony, whereas the spherical *Astrephomene* exposes their flagella by rotating individual cells (Yamashita et al., 2016). Multicellularity arose independently at least twice, once in the family Tetrabaenaceae, once in the common ancestor of the families Goniaceae and Volvocaceae (Lindsey et al., 2021; Pröschold et al., 2018). Cell differentiation has evolved independently at least 4 times in three paraphyletic genera *Astrophomene*, *Pleodorina* and *Volvox* (Lindsey et al., 2021), where sterile somatic cells are reported in all three genera, and large germ cells occur in *Pleodorina* (anisogamy) and *Volvox* (oogamy).

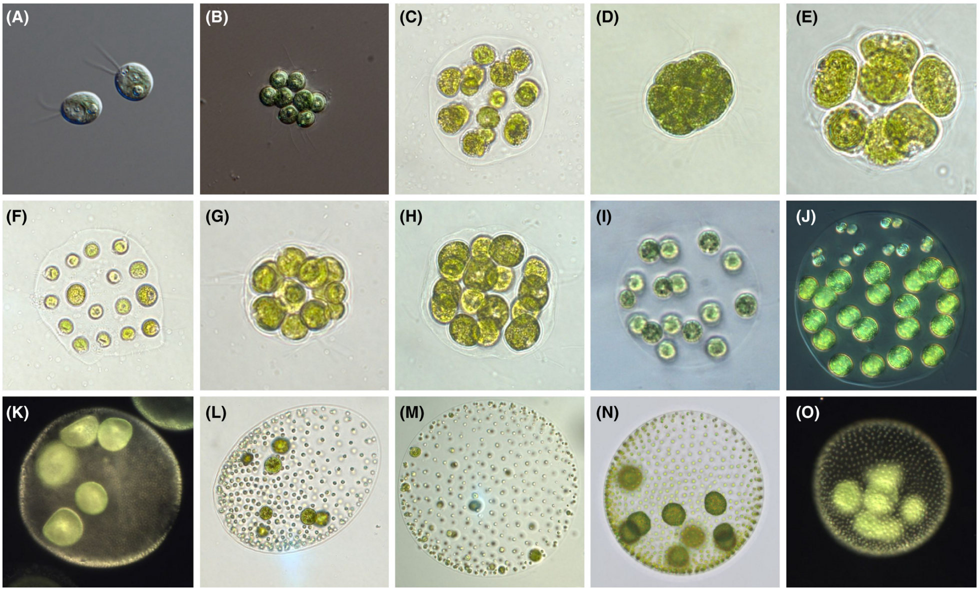

Figure 2. Selected Volvocine algae species

Images of Volvocine species. (A) *Chlamydomonas reinhardtii*, (B) *Gonium pectorale*, (C) *Astrephomene gubernaculiferum*, (D) *Pandorina morum*, (E) *Volvulina compacta*, (F) *Platydorina caudata*, (G) *Yamagishiella unicocca*, (H) *Colemanosphaera charkowiensis*, (I) *Eudorina elegans*, (J) *Pleodorina starrii*, (K) *Volvox barberi*, (L) *Volvox ovalis*, (M) *Volvox gigas*, (N) *Volvox aureus*, (O) *Volvox carteri*. Figure from (Herron, 2016) Molecular Ecology, Volume: 25, Issue: 6, Pages: 1213-1223, First published: 29 January 2016, DOI: (10.1111/mec.13551), Credit for A and B: Deborah Shelton.

Mechanisms of group formation, maintenance, and transformation in green algae

Volvocine algae are one of the best studied clades regarding multicellularity and development. Full genome assemblies are available for *C. reinhardtii* (Merchant et al., 2007), *V. carteri* (Prochnik et al., 2010), *G. pectoral* (Hanschen et al., 2016), *Tetrabaena socialis* (Featherston et al., 2018) and *Astrephomene gubernaculifera* (Yamashita et al., 2021). They have revealed mechanistic changes during the transition from unicellular to multicellular life cycles. For example, genomic work has revealed that the retinoblastoma (*RB*) cell cycle regulator pathway is conserved in algae, plants, and animals (Fang et al., 2006). Functional genomic studies have demonstrated that unicellular *Chlamydomonas* expressing the multicellular *Gonium* variant of the *Rb* gene induced multicellular group formation (Hanschen et al., 2016). This is one of the growing pieces of evidence that many traits linked to multicellularity originated through the co-option of cell cycle related adaptations in unicellular organisms. Genetic control of cell numbers is a hallmark for clonal multicellularity, that is regulated through the ubiquitin proteasomal pathway (UPP).

Enriched genes modifying the UPP were found in the genome of *T. socialis*, consistent with other multicellular volvocine algae (Featherston et al., 2018). *V. carteri* is an established model organism especially suited for the investigation of cellular differentiation thanks to its two distinct cell types, germ and somatic cells. In transcriptomic studies, the germ and soma cell types of *V. carteri* have been shown to have strongly differentiated expression patterns (Klein et al., 2017), with somatic expressions geared towards flagella function and germ cells expressing growth-related genes (Matt & Umen, 2018).

Habitats and the ecological distribution of Volvocine algae

For a system that has been so well studied, knowledge of the natural habitat of volvocine algae is surprisingly limited as systematic sampling efforts are sparse (Herron, 2016). Many volvocine species are allegedly cosmopolitan and commonly found in still water bodies (Umen, 2020). Recently, one large scale study on the community composition of multicellular volvocine algae across the Yangtze River basin concluded that they can be found in rivers and streams but are much more common in lakes and still ponds. The main abiotic factors documented to be associated with their occurrence are water temperature (optimal at 22C), altitude (below 3920m) and total phosphorous (0.06mg/L) (Hu et al., 2023). However, the extent to which species differ in their optimal and tolerated environmental conditions, and if multicellularity influences this, remain unknown.

Selection for multicellularity in green algae

Many potential selective advantages of multicellularity have been demonstrated in volvocine algae. Predation-resistance for instance, has been shown in *Chlamydomonas reinhardtii*, a unicellular species that can facultatively form groups. Multicellular colonies of *C. reinhardtii* were induced under predation of a rotifer species *Brachionus calyciflorus* (Becks et al., 2010) and a filter-feeding ciliate *Paramecium tetraurelia* (Herron et al., 2019). Additionally, the presence of phagotrophic flagellate *Peranema tricophorum* induced higher proportion of multicellular colonies in *Gonium pectorale* (Solari et al., 2015).

Motility is another trait that differs between unicellular and multicellular organisms that is crucial for phototaxis and chemotaxis. The cup shaped *G. pectorale* has reduced motility compared to the spherical *Eudorina* (Solari et al., 2015), and multicellular *V. carteri* is more motile than the unicellular *C. reinhardtii* (Solari et al., 2007). In both cases faster motility was attributed to presumably superior cellular integration of *Eudorina* and *V. carteri*. Interestingly, nutrient uptake can also be improved by better cellular integration. For example, *V. carteri* is among the largest multicellular species of volvocine algae and its greater surface-to-volume ratio decreases the rate of nutrient uptake by diffusion. However, the collective flagella beating increases nutrient transportation from the surrounding media, a process called advection, that compensates for the nutrients lost from reduced diffusion (Solari et al., 2006, 2015).

Nutrients may also be better stored in multicellular algae thanks to their extracellular matrix. One experiment showed that compared to *Chlamydomonas*, *Gonium* had higher relative growth in nutrient poor conditions, suggesting it is better at retaining nutrients (Solari et al., 2015). The explicit link between extra cellular matrix and nutrient retention, however, requires more investigation. Finally, multicellularity is hypothesized to buffer against environmental stressors, and observations of multicellular group formation induced by turbulence (Cornwallis et al., 2023a), sub-optimal pH and deficiency of salt, calcium, and phosphorus have been reported in *Chlamydomonas* and other algae (summarised in (de Carpentier et al., 2019)). This suggests that group formation may be an adaptation linked to coping with environmental stress.

General methodology

Comparative experiments

In Paper I, II & III, we primarily used comparative experiments subjecting multiple algae species (N = 82) to different environmental conditions. In Paper I, 35 species/strains were exposed to predation (planktonic rotifer predator *Brachionus calyciflorus, present or absent)*, nutrient levels (nitrate, high or low) and turbulence (present or absent). Paper two we subjected 38 species to temperature, salinity and nitrogen to phosphorus ratio (N:P). Paper three focused on comparing unicellular algae (N=11) with multicellular algae (N=5) in varying nutrient concentrations and N:P (Fig 3).

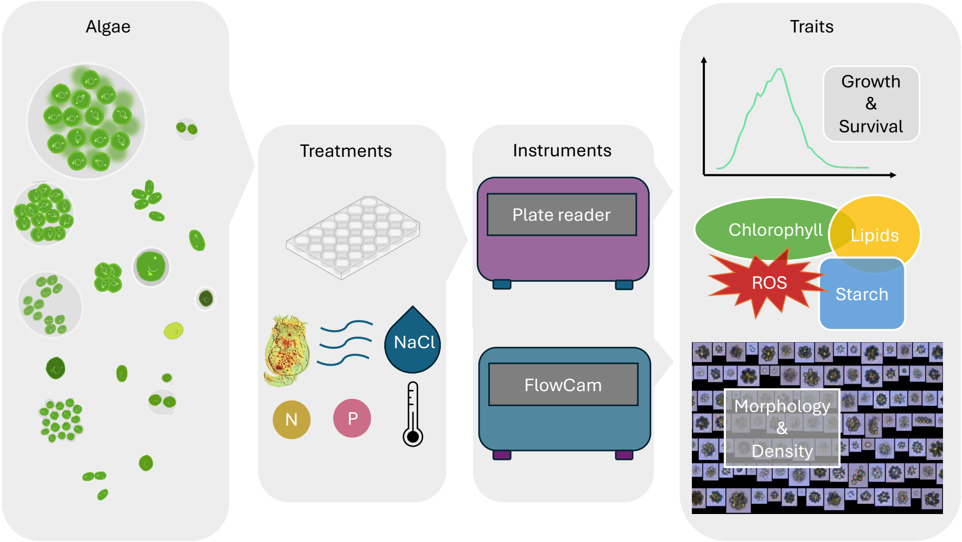

Figure 3. General comparative experimental workflow in this thesis.

Experimental algae were inoculated into well plates or cultural flasks and then subjected to different environmental manipulations including predation (rotifer), turbulence, nutrient level and balance, temperature and salinity. Fluorescence plate reader was used with specific fluorescent probes to obtain traits including growth rates, survival rates (inverse of decline rates), chlorophyll-a, lipids, ROS and starch. FlowCam was used to obtain algal particle densities and morphology, upon which the rates of multicellularity (% cells in multicellular groups) of each species can be quantified. (*Brachionus calyciflorus* image by Takehito Yoshida and Randy O. Wayne - Fig. 1 ofRobinson R (2007) In “Cryptic” Population Dynamics, Absence of Evidence Is Not Evidence of Absence. PLoS Biol 5(9): e255. doi:10.1371/journal.pbio.0050255,CCBY2.5,https://commons.wikimedia.org/w/index.php?curid=2871227)

Algae cultures

Algal cultures were both sampled from natural lakes in Sweden and subsequently isolated and collected from algal culture centeres. They were maintained in 40 ml non-treated culturing flasks (Thermo Fisher Scientific) with breathable cap in a climate chamber with 15°C, 12:12h light: dark cycle and light intensity of 85 µmol photons m−2 s−1. Acclimation was done in in 20°C, 14:10h light: dark cycle with light intensity of 200 µmol photons m−2 s−1. Medium for algal culture were grown in Modified Wright Chu medium (MWC) (Guillard & Lorenzen, 1972), with an addition of 0.002 mg l-1 Na2SeO3•5H2O (MWC+Se).

Morphological analysis

The FlowCam is a flow cytometry platform where samples of algae travelled through a flow cell while a high-speed camera records their images. Algae concentrations can be calculated by the flow rates and number of images. Each image was then used for morphological classification into four categories (single, double, quartet and multi-). Rate of multicellularity, defined as % cells in multicellular groups, was calculated as number of cells in quartet and multi-categories divided by number of cells in all four categories in a sample (Fig 4).

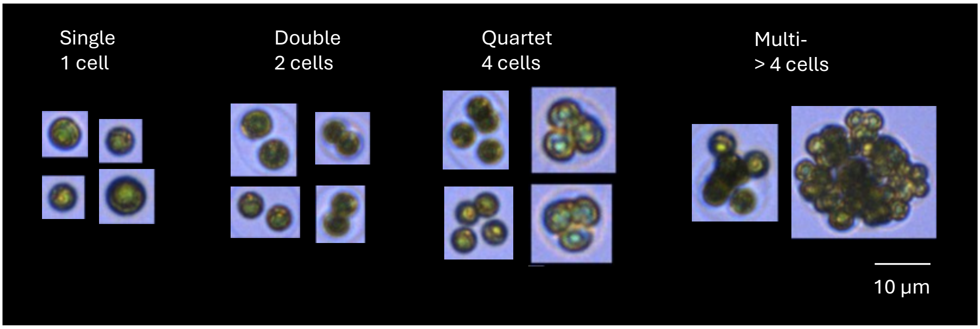

Figure 4. Algal particles classification in FlowCam.

FlowCam images of algal particles showing four morphological categories. Cell numbers from each categories were used to calculate % cells in multicellular groups.

Physiological assays

Physiological traits including chlorophyll-a, reactive oxygen species (ROS), neutral lipids and starch contents were measured using a plate reader. The plate reader has a fluorescence mode that measures the concentration of each compound by exciting it with light at a specific wavelength (excitation). The excited compound then emits light at a different wavelength (emission), and the intensity of this emitted light reflects the compound's concentration. Fluorescence mode was used to measure chlorophyll-a, ROS and neutral lipids (Argyle et al., 2021; Brennan et al., 2012). Starch content was measured using the optical density mode of the plate reader, where the starch in cells were stained by iodine in Lugol's solution and the changing optical density reflects the starch content (Takeshita et al., 2015).

Literature description for algal phenotypes

The national aquatic monitoring scheme in Sweden, financed by the Swedish Agency for Marine and Water Management (SWAM), have been collecting physiochemical and biological data including green algae in Swedish lakes since 1960s. The database (Miljödata MVM, https://miljodata.slu.se/MVM/), hosted by the Swedish University of Agricultural Science (Sveriges lantbruksuniversitet, SLU), provides a great opportunity to study the associations between ecological factors and green algal dynamics.

To study how traits such as multicellularity can influence the distribution and abundance of green algae. I compiled an additional dataset describing the phenotypes of 332 freshwater algae species (114 genera) in Sweden. Traits including multicellularity (yes if typical cell number > 4), presence of extracellular matrix or mucilage, cell number range, size, record of sexual reproduction, etc. The primary sources are the Swedish freshwater algae field guide 'Växtplanktonflora' (phytoplankton flora) and online database 'Algaebase' (www.algaebase.org). Additional sources include other European freshwater field guides and other publications where species were described were used.

Statistical analysis

Data were compiled, and analysed in R (Version 4.2.2) (R Core Team, 2024). We used Bayesian Phylogenetic Mixed Models (BPMMs) for our analysis using R packages MCMCglmm (Hadfield, 2010) and HMSC (Tikhonov et al., 2020). This allows the non-independence of data due to phylogenetic relationships and repeated measures of species across treatments to be properly modelled. Additionally, we used multi-response BPMMs to model the co-evolution of traits among phylogenetically controlled species. This allows the partitioning of within-species effects, among-species and phylogenetic effects (Fig 5). In Paper IV, we used joint species distribution models implemented in HMSC to estimate the effect of multicellularity on the ecological niches species occupy and how different ecological factors influence the presence and abundance of multicellular algae versus unicellular algae.

Figure 5. Illustration of how variances were partitioned in a multi-response Bayesian Phylogenetic Mixed Model.

A) Correlation between two traits within a species. B) Correlation between two traits due to among-species relationships. C) Correlation between two traits among-species due to species that are more closely related have more similar mean trait values. Colours represent species, large points represent the estimated mean trait values of each species, small points represent trait values per species per treatment.

Results

Multicellularity is a generalised response to environmental disturbances

In this thesis, we subject a total 82 algal species to various environmental conditions including predation, turbulence, temperature, nutrients, and salinity. Over 85% of all species showed measurable responses in forming multicellular groups when exposed to environmental disturbances (49 species in Paper II & III shown in Fig 6). This shows that multicellular group formation is a highly plastic trait among green algal species. Specifically, unicellular algae formed multicellular groups in the presence of predation, turbulence (Paper I) and high nitrogen levels (Paper I & III). Additionally, we found that multicellularity increased when conditions deviated from the optimal: multicellularity was induced by cold and warm temperatures, imbalanced N:P ratios and increased salinity (Paper II). The high prevenance of inducible multicellularity in unicellular algae led us to test the idea that multicellularity may be a generalised response when facing adverse environments.

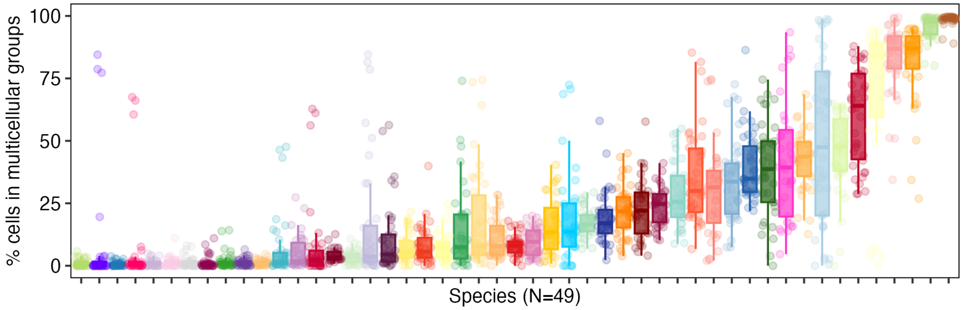

Figure 6. Multicellularity is highly plastic across unicellular algal species.

Unicellular algae can be induced to form multicellular groups across different environments. Species from Paper II (N=38) and Paper III (N=16), five species were the same between experiments in Paper II and III resulting in N(total) = 49. Points represent each species per treatment per replicate, colours represent each unique species.

In Paper II, we tested the generality of multicellular group formation by treating 38 algae in three different environments. We found that plasticity in multicellular group formation in response to changes in temperature (10 to 30 C), N:P (8:1 to 128:1) and salinity (0 to 1 g/L) were correlated across species. This means that species that responded strongly to changes in temperature by increasing the number of cells in multicellular groups also had strong responses to N:P and salinity and vice versa. Such correlated responses to environmental changes did, however, break down under the highest salinity level (2g/L). The reason, interestingly, was that the highest salt concentration accounted for the highest multicellularity in 12 out of 38 species, including two species with very low rates of multicellularity in all other environments (Fig 7). These results suggest that it is more the severity, rather than the type, of environmental stressor that can trigger multicellularity, but that extreme conditions can release novel multicellular phenotypes in some species. Given that multicellularity is highly prevalent, and a multitude of stressors can trigger it, we investigated the physiological states within cells that may be linked to multicellularity.

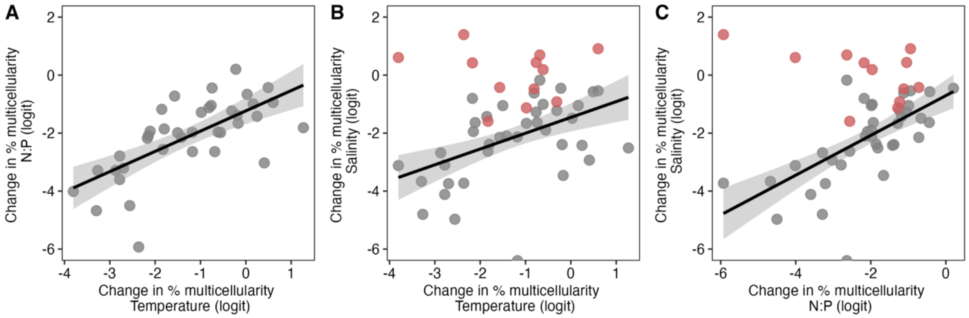

Figure 7. Correlated changes in multicellularity across treatments in Paper II.

A) Temperature versus N:P, B) Temperature versus Salinity, C) N:P versus Salinity. Grey points represent the change in % of cells in multicellular groups per species across pairs of environments, omitting the highest salt level. Red points represent correlations including the highest salt level. Lines and shaded area are linear regression lines with 95% confidence intervals.

Multicellularity likely emerged from single-cell physiological responses

We found links between single-cell physiological traits and multicellular group formation. In Paper I, cells with extra cellular matrix are more likely to form palmelloids (a colony of 4-16 cells embedded in a mucus sheath), and palmelloids formation were significantly linked to multicellular group formation. This suggests a causal relation between extra cellular products and multicellularity. Furthermore, we found positive associations between multicellularity and chlorophyll-a, starch (Paper II) and ROS (Paper II, III, Fig 8). As a key indicator of oxidative stress, ROS can trigger metabolic responses including enhanced starch, lipids and polysaccharide production, which facilitates adhesion between algal cells (Zhang et al., 2023). Taken together, we speculate that the general stress response may result in increased extra cellular matrix and higher multicellularity. Multicellularity may also arise through different group forming mechanisms depending on the severity of the stress: palmelloids (retention of daughter cells) when stress is mild and aggregation when stress is severe (de Carpentier et al., 2019). Palmelloids is a form of clonal multicellularity that has the potential for the evolution of reproductive division of labour due to the relatedness among cells equal to one. In aggregation, however, cells are not guaranteed to be genetically identical with each other. This opens the door for conflict and exploitation within aggregates and limits the potential for evolving further complexity (Fisher et al., 2013).

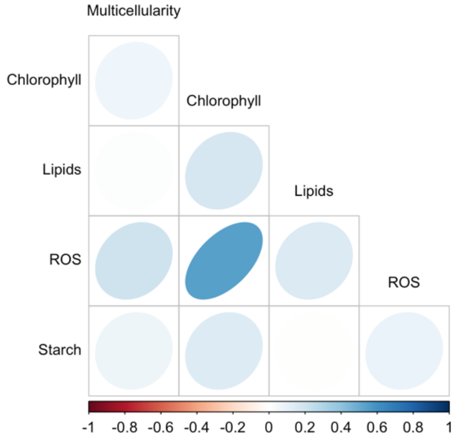

Figure 8. Multicellularity is positively correlated with physiology.

A) visualised model outputs of pair-wise correlations of rates of multicellularity, chlorophyll-a, ROS, lipids and starch. Blue colour indicates positive correlations, colour intensity represents correlation coefficients.

Multicellular algae have reduced growth but enhanced survival

Formation of multicellular groups confers a fitness cost, as multicellularity was associated with slower growth rates in Papers I, II & III. However, we found that multicellularity alters life-history trade-offs in favour of survival. In Paper III, multicellular algae grew slower than unicellular algae across all nutrient treatment conditions, yet they persisted for much longer than unicellular algae (Fig 9). This suggest that multicellular algae are better at enduring deteriorating environmental conditions, potentially due to the internal environments of multicellular groups increasing nutrient recycling and buffering cells against stress. Such heightened survival rates of multicellular algae may also explain the seasonal dynamics between unicellular and multicellular algae in the wild.

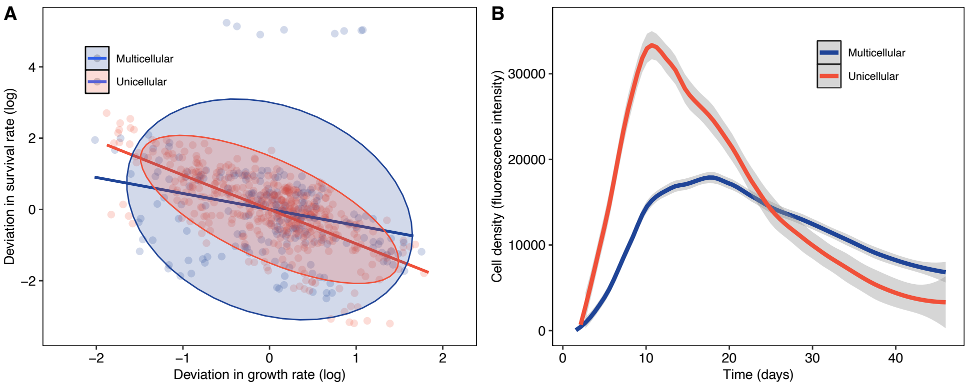

Figure 9. Unicellular species exhibit stronger trade-offs between growth and survival rates than multicellular species.

A) The correlation between growth and survival rates for unicellular and multicellular species. Each dot represents a unique species environment combination; lines are linear predictors fitted by maximum likelihood method and the ellipse areas cover 90% of data points. B) The change in population sizes of unicellular and multicellular species over 45 days, averaged across species and environments (lines were drawn by locally estimated scatterplot smoothing (LOESS) , degree of smoothing span = 0.4, shaded areas represent standard error.)

Different niche for multicellular and unicellular algae in the wild

The widespread coexistence among algae with different morphology in the wild has long been a fascination and focus in algal ecology research (Burson et al., 2019; Hutchinson, 1961; Roy & Chattopadhyay, 2007; Tilman, 1977). Multicellularity changes algae’s interaction with the environment due to increased size, establishing internal environment and potentially improved locomotion (Solari et al., 2007) – factors that may be favoured in different ecological niches. To address this issue, we characterised the ecological niches in 114 genera of green algae (Chlorophyta) in 616 Swedish lakes (Fig 10A). We found strong seasonal differences between unicellular and multicellular genera (Paper III & IV): multicellular genera are much more represented during summer months compared to other seasons, whereas the presence of unicellular genera is more even across the year. Additionally, higher total phosphorus level and temperature increases the prevalence of multicellular genera more than unicellular genera (Paper IV, Fig 10 B-D). Taken together, we find evidence for niche differentiation between multicellular and unicellular algae over large spatial and temporal scales.

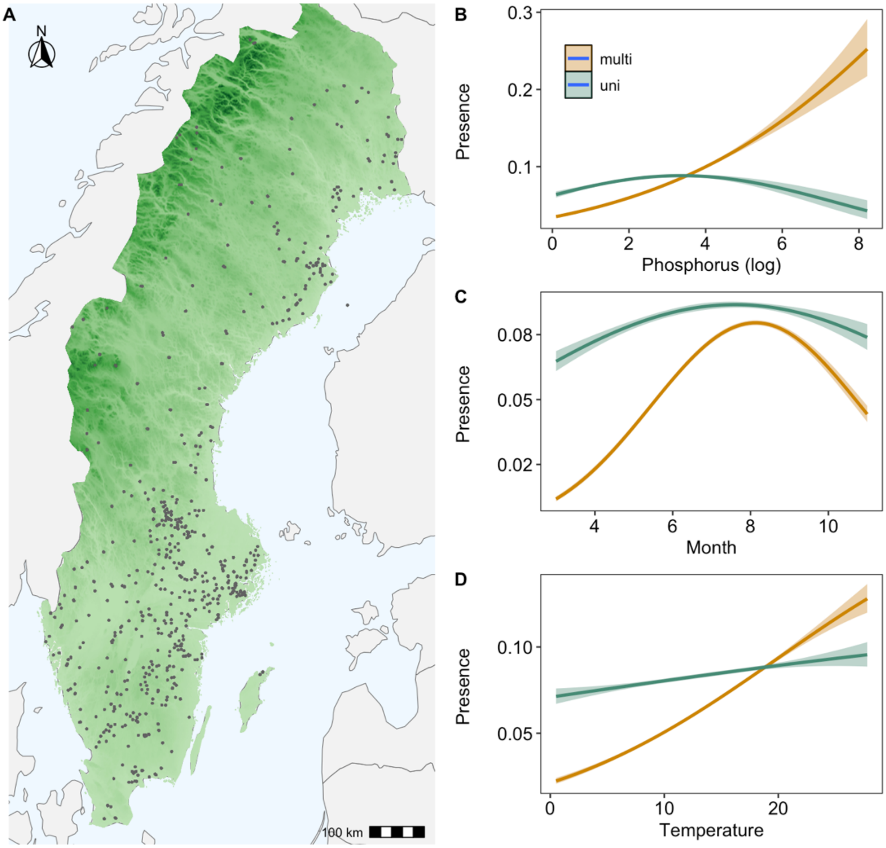

Figure 10. Ecological niche differentiation between unicellular and multicellular algae in Swedish lakes.

A) Map of lakes in Sweden where the ecological niches of unicellular and multicellular algae were studied. B-D) The presence of unicellular and multicellular algae are differentially influenced by phosphorus, seasonality (month) and temperature.

Conclusion and future directions

This thesis demonstrates that freshwater algae frequently form multicellular groups in response to different environmental disturbances. This is likely due to the fact that multicellularity is a product of physiological responses to stress. Unicellular algae forming multicellular groups generally conferred a reproductive cost, but benefits were more difficult to detect. Studying multicellular algae, however, showed that they shift to a slower life-history strategy characterised by slower growth and longer survival. This change has the potential to explain the distribution of multicellular and unicellular algae in the wild.

Using comparative approaches, we were able to elucidate broad patterns and establish general links between multicellularity and physiological processes in freshwater algae. There are numerous suggestions of how multicellularity can evolve by co-option of pre-existing unicellular functions (A. M. Nedelcu & Michod, 2006; Ruiz-Trillo et al., 2023; Sebe-Pedros et al., 2011). But many of such proposed functions in unicellular organisms remain unknown. Future research can benefit from zooming into these physiological links, with special focus on how the environment can change cell cycles in unicellular algae and in other organisms; and compare such mechanisms across a wider sampling across taxa.

In unicellular organisms such as Chlamydomonas reinhardtii, the cell cycle is a temporal sequence of stages of growth, DNA synthesis, mitosis, quiescence (dormancy) and sexual differentiation (Bisova et al., 2005; Bišová & Zachleder, 2014; Cross & Umen, 2015; Hallmann, 2011; Takeuchi & Benning, 2019). How environment can impact these different stages has meaningful implications for the evolution of multicellularity. For example, nutrients and light determine the length of the growth phase in multiple fission algae, and larger cells will create larger multicellular palmelloids (Kirk, 2003, 2005; J. Umen & Herron, 2021). The environment can also change the production of extra cellular matrix, which can prolong the retention of daughter cells (Cornwallis et al., 2023b; de Carpentier et al., 2019). This may increase the length of the multicellular phase of the unicellular cell cycle, exposing variation in multicellular groups to selection. If we expand this reasoning to a wider range of taxa, we can eventually build up a clearer picture of the evolution of multicellularity: how different unicellular taxa, with their unique physiology and ecological conditions, find their unique routes to multicellularity (Niklas & Newman, 2020).

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Algal doodle masterpiece by the lovely folks in the department of biology, Lund University

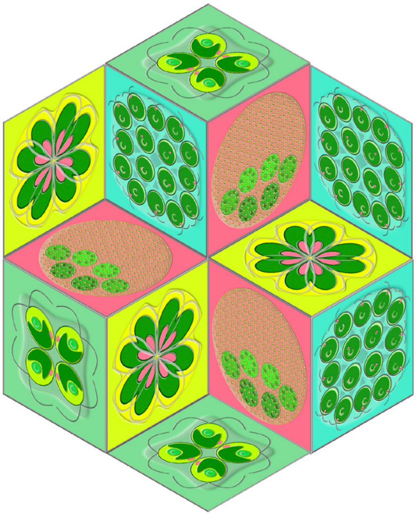
List of Papers

I. Cornwallis, C. K., Svensson-Coelho, M., Lindh, M., Li, Q., Stábile, F.,Hansson, L.-A., & Rengefors, K. Single-cell adaptations shape evolutionary transitions to multicellularity in green algae. *Nature Ecology & Evolution.* 7, 889–902 (2023).

II. Li, Q., Isanta-Navarro, J., Svensson-Coelho, M., Hoogenboom, R., Milocco, L., Uller, T., Rengefors, K., Cornwallis, C. K. Multicellularity emerges as a general response to extreme environments across unicellular green algae. *Manuscript.*

III. Li, Q., Svensson-Coelho, M., Rengefors, K., Cornwallis, C. K. Nutrient dependent life-history strategies explain ecological differentiation between unicellular and multicellular green algae. *Manuscript.*

IV. Li, Q., Svensson-Coelho, M., Alling, T., Rengefors, K., Cornwallis, C. K. Ecological niche differentiation between unicellular and multicellular green algae. *Manuscript*.



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