THE SHIFTING ROLE OF CELL DIVISION DURING AN EVOLUTIONARY TRANSITION TO MULTICELLULAR-LEVEL INDIVIDUALITY

by

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SIGNED: Deborah Ellen Shelton

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DEDICATION

To my parents, who have supported and inspired my academic journey in countless ways.

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ABSTRACT

During the transitions from unicellularity to multicellularity, cells transitioned from functioning as wholes to functioning as parts of wholes. In the colonial freshwater green flagellates known as volvocine algae, living "intermediate form" species give ample evidence concerning how cells gradually lost autonomy and began functioning as dedicated parts. This dissertation concerns how and why the role of cell division changed in unicellular to colonial volvocine algae. We review a recent book on levels of selection and apply a proposed three-stage transition to the example of volvocine algae. We found that, in contrast to the previous description of "stage 1", the concept of group reproduction is potentially applicable to very early-branching colonial volvocine algae. This possibility indicates that the role of cell division could have shifted (to function in group reproduction) earlier than was previously thought (Appendix A). We show that, given some reasonable assumptions, cell- and colony-level fitness are equivalent in undifferentiated colonial volvocines (Appendix B). In spite of this, our models show that cell division number could evolve in response to specifically colony-level factors. Cell division number could be regulated indirectly via allocation to growth (Appendix B) or directly via regulation of the growth-to-first division transition (Appendix C). The extent to which group factors matter in the outcome of selection on cell division number is a matter of degree and is quantifiable (Appendix B). Colony cell number could be a genuine group-level adaptation, even in the simplest volvocine algae (Appendix B and C). Because a size-dependent growth trajectory is a substantial group-level cost of higher division numbers, our analysis highlights the potential importance of understanding how colony size affects cell growth (Appendix C). We also present data on cell-type allocation in *Volvox* (Appendix D). The *Volvox* colony is clearly the level of function for cell divisions and cell fate acquisition. However, this work indicates that the

precision with which *Volvox* development attains these colony-level goals may be low compared to more complex multicellular organisms.

INTRODUCTION

Explanation of the problem and its context

Darwin (1859, p. 196) wrote:

> If a working ant or other neuter insect had been an animal in the ordinary state, I should have unhesitatingly assumed that all its characters had been slowly acquired through natural selection; namely, by **an individual having been born** with some slight **profitable modification of structure**, this being **inherited by its offspring**, which again varied and were again selected, and so onwards. (emphasis added)

Darwin's succinct specification of what is meant by "natural selection" has resonated with many biologists and philosophers, who conceptualize natural selection as the logical consequence that follows when a few preconditions are met. The exact nature of the preconditions are subject to much interpretation, the details of which are not critical here (Lewontin 1970; Maynard Smith 1987; Okasha 2006; Godfrey-Smith 2008). The preconditions are generally of the following form. There must be discrete things, call them individuals, with a life cycle—i.e. things that are born and that are capable of becoming parents. In addition, there must be more than one of these so-called individuals (i.e. they form a population) and they must vary in heritable traits that are relevant to production of offspring. Given that individuals in these senses exist, natural selection occurs. Necessary for the concept of natural selection are the concepts of discrete things, of parent-offspring relationships among these things, and of heritable traits that cause more or less production of offspring.

It is clear why Darwin, in the above quote, was concerned about the case of sterile worker insects. Since sterile insects do not produce offspring, the traits that make them sterile could not have evolved by natural selection, conceptualized as described above. But the traits that make

them sterile hardly seem accidental, so non-selective explanations of them are also not satisfying. Even without the benefit of knowing modern genetics, Darwin was able to see the essence of the solution to the dilemma raised by sterile insects. He wrote, "This difficulty, though appearing insuperable, is lessened, or, as I believe, disappears, when it is remembered that selection may be applied to the family, as well as to the individual, and may thus gain the desired end." (Darwin 1859, p. 196)

Now, about a hundred and fifty years after Darwin wrote about the "special problem" of the neuter insects, the solution that he envisioned has been fleshed out considerably. It is evident that the sterility phenotype of worker eusocial insects evolved in response to the "desired end" of increasing worker inclusive fitness or of increasing colony fitness. The two ways of framing the solution to the dilemma (inclusive worker fitness or colony/group fitness) are—in some cases, at least —a matter of different perspectives and of different mathematical formalisms capturing the same underlying processes (Queller 1992; Lehmann and Keller 2006; Lehmann et al. 2007). (But also see van Veelen et al. (2012) and Simon et al. (2013).) Far from simply closing the book on one particular problem case, the solution to the "neuter insect dilemma" has opened up new areas of inquiry.

Consider in more detail the "group/colony fitness" approach to resolving the dilemma. Natural selection is a process that occurs when certain preconditions are met. These preconditions are specified with respect to "individuals" within a population. Yet, it is not necessary for such an evolutionary individual to be one specific type of thing. An individual could be a prion, a synapse, a cell, an ant, or a "family" of ants, to name only a few. Perhaps a word or an idea could even be an evolutionary individual. Thus, the dilemma of neuter insects can be solved if the colony/group of insects containing the neuter is the relevant evolutionary

individual. This simple observation—that a variety of types of things can be "individuals" in the sense that is relevant for natural selection—has a multitude of ramifications and raises new questions.

One set of ramifications concerns a specific way in which some new evolutionary individuals come about. The logic of natural selection can be applied to a certain kind of thing (an ant) as well as to groups of that kind of thing (a family of ants). So the formation of groups of individuals that acquire—at the group level—the properties necessary to fit as "individuals" in the logic of natural selection is one source of new evolutionary individuals. That is, new wholes can originate when previously-existing wholes become mere parts. The history of life—with endosymbiosis leading to eukaryotic cells, a multitude of origins of multicellularity (Herron et al. 2013), and the evolution of eusociality—is replete with examples of this kind.

Are there generalities about how and why evolutionary individuals sometimes evolve into parts-of-higher-level-individuals? What specific properties of things allow them to work as "individuals" in the logic of natural selection? How do the properties that make a thing an individual relate to the properties that might make a group of those things an individual? Do these individuality-affecting properties come in degrees and, if so, how and to what extent do things that are individuals-to-a-degree evolve adaptations? See Godfrey-Smith (2011) and Bouchard and Huneman (2013) for a recent books that explore these and related questions. These are the larger, motivating questions behind this work.

As scientists and philosophers have followed the thread of inquiry articulated in Darwin's thoughts about neuter insects, new vistas in need of exploration have opened up. There is the challenge to understand natural selection as a truly general phenomenon that occurs in diverse kinds of evolutionary individuals. There is the challenge to explain the repeated emergence and

evolution of hierarchically nested biological individuals. There is the challenge to understand simultaneous natural selection on multiple, interrelated substrates (e.g. multicellular bodies and the cells within them).

In this dissertation, I address these topics by studying the shifting role of cell division in volvocine algal species that straddle the divide between unicellular-level and multicellular-level selection. This topic unifies the appended papers and manuscripts. Appendix A largely centers on the problematic notion of "reproduction" and how to apply it during an evolutionary transition. Okasha's (2006) three-stage schema can be interpreted in terms of reproduction: in stage 1 it exists only at the lower level, in stage 2 it exists at the higher level but is fully coupled to reproduction at the lower level, and in stage 3 it exists primarily at the higher level. The role of cell division at the start of a transition is to reproduce cells, so the discussion of levels of reproduction is directly relevant to the unifying theme of this dissertation. Appendix B focuses more explicitly on cell division by developing a model of cell and colony fitness as a function of cell allocation to growth. In this section, cell allocation to growth is closely linked to cell division number by the assumptions that growing time is fixed and final size determines cell offspring number. The trait of cell allocation to growth is used as an example to explore issues of shifts in the level of selection and adaptation. The points developed in Appendix B apply more generally than just to the particular trait of cell allocation to growth/cell division number. However, this section is closely related to the theme nonetheless because methods to distinguish the level of selection acting on a trait are needed to parse how and when the role of cell division changes from simply being cell reproduction. Appendix C explores the issue of cell division number with more detailed modeling of both how and why unicellular number of divisions per cycle is regulated. In both Appendix B and C, final cell size determines number of divisions per

cycle. By assumption in Appendix B, a cell's growing time is fixed and a cell can only increase its final size (and thus cell division number) by increasing its endogenously-controlled allocation to growth. In contrast, in the models in Appendix C, the assumption of fixed growing time is relaxed and allocation to growth is instead assumed to be constant. By assumption in Appendix C, a cell can only increase its final size (and thus cell division number) by an increasing its duration of growth. Growth rate of course still affects final size, but in Appendix C, growth rate is assumed to be completely determined by the environment (i.e. there is no endogenously-controlled allocation to growth in Appendix C). This manuscript also begins to address some of the fundamental factors that could have changed the costs and benefits of regulating cell divisions in a particular way, given that a rudimentary colonial life cycle has evolved. Finally, Appendix D addresses the unifying theme by asking how *Volvox* developmental features relate to the variability of cell-type allocation. Re-purposing cell divisions so that they adaptively allocate cells to two discrete tasks/morphologies is a major innovation driven by higher-level selection in *Volvox*. We studied the precision of these developmental outcomes (cell type allocation) with the goal of beginning to understand how and to what extent higher-level selection has been successful at accomplishing this re-purposing.

"Volvocine algae" is an informal name for a clade of freshwater, photosynthetic flagellated green algae that includes colonial species such as *Volvox carteri* as well as unicellular species such as *Chlamydomonas reinhardtii*. Here we focus on the asexual portions of the lifecycle of these algae, although they are facultatively sexual. Useful summaries of basic information about this group can be found in Coleman (2012) and Nozaki (2003). Cell division is an illumining trait to focus on because the role of cell division clearly changes as a higher-level (multicellular) evolutionary individual emerges. For example, in unicellular *C. reinhardtii*, cell

divisions function to reproduce the evolutionary individual, i.e. the cell. In multicellular *V. carteri*, some cell divisions produce tiny somatic cells along with larger reproductive cells. So cell division in this case clearly functions in the development of the evolutionary individual (i.e. the colony) as well as in its reproduction. The questions of how and why selection shifts from being in effect primarily at the lower level to being in effect primarily at the higher level is paralleled by the questions of how and why the function of traits shifts from increasing lower-level fitness to increasing higher-level fitness. In this dissertation, I investigate the trait of cell division in a diversity of volvocine algae that are thought to represent grades of higher-level individuality. Investigating how the function of this trait shifts levels gives a concrete foothold that helps in framing and making progress on questions concerning the slipperier, abstract issues surrounding shifts in levels of fitness and selection.

Explanation of the dissertation format and nature of collaborative work

This dissertation includes four appendices, each formatted as an independent manuscript. Appendix A is a published paper (Shelton and Michod 2009) that reviews *Evolution and the Levels of Selection* by Samir Okasha. This paper also explores some applications of Okasha's ideas to volvocine algae. Appendix B is a manuscript that is in press at *Biological Theory*. This manuscript uses modeling approaches to address an issue at the interface of biology and philosophy: the problem of distinguishing the effects of group selection versus so-called "fortuitous" group benefits (Williams 1966). Appendix C is a manuscript that is in preparation. In it, I use modelling approaches to examine how and why volvocine algae cells might regulate the number of cell divisions that occur rapidly back-to-back during a single asexual cell cycle. I make extensive use of published data in order to promote connections with empirical research. Appendix D is a published paper (Shelton et al. 2012) that presents some new empirical data on

Volvox cell-type allocation. Cell division in *Volvox* embryos functions to allocate cells to different tasks. The analysis of these data includes development and exploration of predictions from a basic model.

This section provides the context of the problems that unify the appendices. The "Present Study" section summarizes each appendix in more detail and describes the major findings and conclusions of the dissertation as a whole. The appended papers and manuscripts are collaborative works, and my role was primary author/researcher for all of them. In addition to writing these works, I led the statistical and mathematical modelling portions of the work.

PRESENT STUDY

The methods, results and conclusions of this research are presented in the manuscripts appended to this dissertation. This chapter is a summary of the research presented in the appendices and also provides the overarching conclusions of the dissertation.

The 2006 book *Evolution and the Levels of Selection* by philosopher Samir Okasha has gone a long way towards clarifying past levels-of-selection debates and setting up a common framework for making progress on substantive levels-of-selection issues. Appendix A is a review of this book and a first pass at applying some of the ideas within it to a concrete biological example of a shift in the level of selection, namely the evolution of multicellularity in volvocine algae. We find that Okasha's (2006) idea of a generalized three-stage transition did not extend easily to the volvocine algae. With respect to the establishment of unambiguous group-level parent-offspring lineages, the volvocine algae seem to have made the transition to a higher level almost immediately, essentially by-passing the earliest stage. Okasha (2009) pointed out that our critique is relevant to a distinction between modes of group formation (aggregation vs. cohesion of offspring). In response to our paper, Okasha writes: "Perhaps a single overarching schema, of the sort I was hankering after, cannot subsume these two different sorts of evolutionary transition." He also writes:

> If Michod and Shelton are right that with an auto-colonial mode of colony formation, the notion of group reproduction can apply at the earliest transitional stages, as soon as colonies start to form, then it follows that stage one of my schema will be not be universally applicable to evolutionary transitions. I suspect that they are right on this point, though for the argument to be fully conclusive, one would need a principled account of what 'group reproduction' amounts to.

In lieu of focusing explicitly on producing "a principled account of what 'group reproduction' amounts to", in the remaining appendices I get at the issue of group reproduction in a more indirect manner. Because cell division *is* cell reproduction in unicellular species, understanding how and why the role of cell division changed during a transition contributes to understanding how "reproduction" (in the sense relevant for natural selection) shifted to a higher hierarchical level.

In Appendix B, we address the challenge of identifying "genuine" group selection by developing an explicit, volvocine-algae-inspired model for what group fitness would be in the absence of group-level relationships between a trait (cell division regulation) and group fitness. We call this "counterfactual fitness" because in many actual cases of interest there are group-level relationships between traits and group fitness. The idea behind counterfactual fitness is to capture the effects of the group life cycle alone (without simultaneous group-level effects of the trait in question), so that it can be properly accounted for. We present a basic model of unicellular and colonial fitness as a function of cell allocation to growth. (Because here we assume fixed growing time and dependence of cell division number on final size, this trait is determines the number of rounds of divisions per cycle and, therefore, cell offspring number.) This model is used to explore the interrelationship of fitness at the two levels and to explore the effects of different assumptions on whether a trait value is genuinely adaptive specifically at the group level. We use the Price equation and the concept of counterfactual fitness to partition trait change into change due to group-dependent cause-effect relationship between a trait and fitness and change due to group-independent cause-effect relationship between a trait and fitness. This suggests that three distinct categories of selection ("global individual level selection", "within-group selection", and "genuine group selection") could occur when there are two hierarchically

nested levels. We consider only two categories ("global individual level selection" and "genuine group selection") and discuss difficulties with the prospects of extending this analysis to cover all three categories. This analysis shows how understanding the function of traits from an optimality perspective sheds light on the level at which traits are adaptive. By extension, because selection is the only cause of adaptations, knowledge of the hierarchical level(s) of adaptations implies knowledge of the hierarchical level(s) of selection. That is, "genuine" group selection can be recognized by its effect, group-level adaptations. (Of course the inverse inference—i.e., from a lack of group adaptations to a lack of group selection—should not be made.)

In Appendix C, we develop a more detailed optimality analysis of regulation of cell division in unicellular and simple colonial volvocine algae. In contrast to Appendix B, here we assume that growing time can vary among individual cells whereas cell growth rate is fully determined by the environment (i.e. no endogenously-controlled allocation to growth). We delve into adaptive hypotheses for unicellular *Chlamydomonas reinhardtii* cell cycle regulation because this sets the stage for later changes in the level at which cell cycle regulation is adaptive. We develop models for both how and why regulation of *C. reinhardtii* cell divisions (specifically, the number of rounds of division per cycle) could be adaptive. Our work highlights the importance of understanding this trait as a norm of reaction, that is, a predictable change in trait value as a function of an environmental parameter. We also begin to develop ideas about how fundamental size scaling relationships might come into play as cell division regulation first begins to be selected at a higher level of organization. Our analysis suggests that the early changes in the regulation of number of divisions per cycle in volvocine ancestors might have been adaptive specifically at the group level. Throughout Appendix B and C, we develop insights

into the relationship between selection and adaptedness at particular level(s) with an eye towards applying these concepts in a way that is concrete and amenable to empirical investigation.

Finally, in Appendix D, we move our attention away from the species in which application of individuality concepts is murky. The focus of Appendix D is patterns of cell division in definitively multicellular *Volvox* species. Just as sterile insects contribute to the fitness of insect colonies, sterile somatic cells contribute to the fitness of *Volvox* colonies. In these species, the colony is unambiguously a unit of selection, so cell divisions function in the development of embryonic colonies. (Unlike in some other organisms, cell divisions in *Volvox* do not contribute to the growth of colonies.) However, even when colony-level individuality is unambiguous, the challenge of re-organizing cell division patterns in the service of higher-level goals is substantial and the ways in which this challenge is met are still poorly characterized. We are particularly interested in the potential for selection to canalize (i.e. lower the variation in) a higher-level phenotype, namely allocation to somatic versus reproductive cells. We observed cell-type allocation in clones of developmentally diverse *Volvox* species in one environment and found distinct differences among species. This study is a first in-depth look at the variational properties of cell-type allocation in *Volvox* species, and so far connecting patterns in cell-type allocation variation to specific developmental features has proven difficult. Nonetheless, our characterization of species differences in cell type allocation variation is a step that would facilitate future comparative studies. Additionally, this study supports a more general picture of variability in *Volvox* cell type allocation. The observation of high intrinsic variability (low canalization) in *Volvox* compared to more complex multicellular organisms supports the view that a general feature of transitions could be low canalization of developmental outcomes when a new type (level) of organism first originates followed by selection for higher canalization. Other

explanations (e.g. adaptive bet hedging in *Volvox*, some sort of constraints. etc.) are certainly still plausible; the idea of increased developmental canalization during a transition is only one possible explanation of the data at this point.

Several conclusions emerge from the work in this dissertation. Our first motivating question is: are there generalities about how and why evolutionary individuals sometimes evolve into parts-of-higher-level-individuals? This work concerns only one example of such a transition (the evolution of multicellularity in volvocine algae), but nonetheless suggests something about this more general question. A major conclusion of Appendix A is that the general scheme proposed by Okasha (2006) did not fit well with volvocine algae because the palintomy/autocolony connection allows for the very early emergence of group-level lineages. Thus, the distinction between groups that form by aggregation versus those that form by cohesion of offspring is an important one to keep in mind when thinking of generalities. A major conclusion of Appendices B and C is that changes in cell trait values in response to group-specific factors may have occurred very easily/quickly for traits that were regulated in response to external environmental factors in the unicellular context. Adjustment of these trait values could in turn contribute to selection for group cohesion, thus solidifying the group-level life cycle. Although speculative at this point, this kind of scenario could generalize to other transitions. Also, the lack of importance of conflict within the group at the early stages of a transition could generalize to other study systems, particularly when group formation occurs through the cohesion of offspring.

Our second and third motivating questions are: what specific properties of things allow them to work as "individuals" in the logic of natural selection? How do the properties that make a thing an individual relate to the properties that might make a group of those things an

individual? In this work, we see how a rudimentary group life cycle could occur even if groups are not "individuals" in the evolutionarily relevant sense of the word (Appendix B). The palintomic cell cycle (in which a long period of growth is interspersed with rapid divisions) and the possibility of cells adhering to each other (or to parent cell wall material) are the cell properties relevant to the emergence of a rudimentary group life cycle in volvocine algae. A group life cycle does not necessarily imply group selection. Rather, distinguishing "genuine" group selection (and, thus, genuine group-level individuals) requires careful attention to the hierarchical level of the relationship between traits and fitness. This point was emphasized by Heisler and Damuth (1987) and comes out of our work as well. We explore in depth how a cell property (number of cell divisions per cycle) that affects unicellular fitness can affect group fitness, given that a group life cycle emerges from small changes in the properties of those cells (i.e. temporary adhesiveness of offspring cells). The sources of group-level effects on fitness, e.g. a relationship between colony cell number and cell mortality risk (Appendix B) or a relationship between colony cell number and cell growth (Appendix C), are the roots of group-level individuality.

In relation to volvocine algae in particular, our work indicates that regulation of number of cell divisions is likely to be an important life history trait in unicellular species and that this trait is extremely closely related to body size and generation time/growing time. The adaptive regulation of this suite of traits is a complicated problem in unicells, and it is likely that the costs or benefits related to this optimization problem changed as soon as an autocolony (group) lifecycle emerged from a palintomic (unicellular) cycle. This is significant because traits that are regulated differently in response to specifically group-level costs or benefits are the products of "genuine" group selection and cannot be dismissed as "fortuitous group benefits". The upshot is

that genuine group selection may have effected trait change (change in regulation of divisions) in the earliest-branching colonial volvocine algae, calling into question their status as representatives of an early stage in which full multicellular individuality has yet to emerge.

Our final motivating question is: do these individuality-affecting properties come in degrees and, if so, how and to what extent do things that are individuals-to-a-degree evolve adaptations? In Appendix B, we conclude that for a specific trait (given no within-group selection), the effects of global individual selection versus genuine group selection is a matter of degree and can be quantified. We introduce the concept of "counterfactual fitness" in order to make explicit the fitness that a given genotype would have if group structures and life cycles existed but had no relevant effects. An explicit model of this sort is needed to recognize trait change that is due specifically to selection at the group level. Extensions of this work to cases that include within-group selection and multiple traits would be needed to continue to make progress on the larger question of degrees of individuality and the relative strength of different levels of selection.

Finally, issues of the extent to which emerging individuals evolve adaptations was also addressed in Appendix D. In *Volvox*, the presumed "full" multicellular individual, this work supports the view that the precision of their developmental outcomes is lower than is typically seen in other multicellular organisms. We also concluded that precision of developmental outcome was species-specific, though how these differences related to among-species differences in development is still not clear. Although other explanations are possible (e.g. adaptive low precision, as in bet-hedging), the result of high variance in *Volvox* phenotype at least suggests that multicellular-level selection could be less effective (at lowering variance in developmental outcome) in *Volvox* compared to other organisms. That is, the degree of higher-level adaptedness

might be comparatively low in *Volvox*. This work highlights the connection between the evolution of mechanisms of higher-level development (ontogeny) and the evolution of individuality at the higher level.

To some, the concept of individuality might seem hopelessly fuzzy, and this viewpoint might inspire a strategy of avoiding the topic altogether. But the issue of refining the biological individuality concept should not be swept completely aside because the very logic of evolution by natural selection presupposes a coherent concept of individuality. Thus, there are biological issues that hinge on developing this concept. A direct answer to the question: "what is this individuality concept that is needed for the logic of natural selection?" is difficult. One might start by saying that individuals in this sense must be separable, capable of begetting offspring that resemble themselves, etc. But what is really meant by criteria of that sort? Are these particular criteria really the ones that matter? How should one measure these criteria in practice? Michod (1999, p. 107) takes this kind of approach by writing that "Darwin argued (1859) that natural selection requires heritable variation in fitness" and "Levels in the biological hierarchy…possess these properties to varying degrees, according to which they may function as evolutionary individuals or units of selection." The justification for and application of these sorts of criteria are not straightforward. For example, the issue of "fortuitous group benefits" indicates that some groups, in spite of having what appears to be heritable variation in fitness, are not true "individuals" in the sense that biologists want to use the concept. Additionally, employing the concept of "fitness" to define/explicate the concept of "individual" opens more issues because the concept of fitness itself is difficult and controversial (Ariew and Lewontin 2004).

I approach the issue in a different, indirect way. Rather than asking: "what is this individuality concept that is needed for the logic of natural selection?", I ask: "what evidence is

there that a particular thing is or is not an individual in the sense needed for natural selection to work?" I use as a starting point the facts that natural selection is the only known mechanism for producing the appearance of good design (adaptations) and that natural selection generally will produce the appearance of good design in the absence of other factors (e.g. gene flow from outside the system in question, too-low variation). This allows for the possibility of (at least temporarily and under some circumstances) setting aside the issue of how/why a particular thing is an individual in the sense relevant to natural selection and simply observing whether or not it is. Group-level good design implies effective group-level natural selection, which in turn implies that the groups in question must (somehow) be individuals, at least to a degree. Of course lack of group adaptations are less informative as to the individuality status of the group.

The comments quoted above notwithstanding, it is tempting to think that a particular thing either does or does not work in the logic of natural selection (with no or very little middle ground). Whether or not that statement is true, it is clear that ample middle ground in the effectiveness of natural selection at producing well-designed traits is possible. Thus, focusing on the products of natural selection (well-designed traits) rather than the logic of natural selection meshes well with the commonly-held views that biological individuality comes in degrees and that groups with only "fortuitous group benefits" are not individuals. Furthermore, grades in the effectiveness of natural selection seem to line up rather well with grades in properties that some think should be related to a well-delineated and justified concept of "individuality". For example, many of the individuality criteria explored by Godfrey-Smith (2011) (e.g. fidelity of inheritance, dependence of realized fitness differences on intrinsic properties, smoothness of the fitness landscape, bottleneck, reproductive specialization, overall integration) clearly relate to how effective a particular population of purported individuals is at evolving good design. I think that

philosopher Sterelny (2011) was right in observing that: “While Godfrey-Smith does not use the term, in part *Darwinian Populations and Natural Selection* is an essay on evolvability.” My view is also consistent with Michod’s (1999, p. 17) idea that in addition to having properties that allow it to be a unit of selection, a higher-level individual must also have mechanisms to modulate lower-level change. Modulation of lower-level change is one way that higher-level individuals can respond more effectively to higher-level selective pressures.

According to the work presented here, it is quite possible for very simple, poorly integrated colonies to have traits that are adaptive specifically at the group level, thus to have some degree of individuality. That is, the emergence of a rudimentary group-level life cycle (where nearly-identical offspring cells stay together for some time before splitting from one another and then undergoing cell division) is sufficient for some level of effective, genuine group selection. However, this work also emphasizes that when new individuals (colonies) are created because former individuals (cells) evolve into mere parts, the early emergence of a group life cycle alone is not sufficient to cause a rapid and complete shift from cells-as-wholes to cells-as-parts. The differences in group-level cohesion and in the sophistication of group-level design among the living “intermediate form” volvocine algae are still relevant indicators of a gradual change from cells-as-wholes to cells-as-parts, i.e. a gradual shift in the level at which selection is effective/ the level of individuality.

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APPENDIX A. PHILOSOPHICAL FOUNDATIONS FOR THE HIERARCHY OF LIFE

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Philosophical foundations for the hierarchy of life

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Abstract We review *Evolution and the Levels of Selection* by Samir Okasha. This important book provides a cohesive philosophical framework for understanding levels-of-selections problems in biology. Concerning evolutionary transitions, Okasha proposes that three stages characterize the shift from a lower level of selection to a higher one. We discuss the application of Okasha's three-stage concept to the evolutionary transition from unicellularity to multicellularity in the volvocine green algae. Okasha's concepts are a provocative step towards a more general understanding of the major evolutionary transitions; however, the application of certain ideas to the volvocine model system is not straightforward.

Keywords *Chlamydomonas* · *Eudorina* · Evolutionary transitions · *Gonium* · Individuality · Multicellularity · Multilevel selection · *Pleodorina* · Volvocaceae · *Volvox*

Evolution by natural selection is the cornerstone of modern biology, but Darwinian principles (i.e., the conditions under which natural selection occurs) are abstract and general. Darwinian processes underlie the immune system and may apply to self-organization of the brain (Edelman 1987; Michod 1989); they are not even restricted to living systems (Bernstein et al. 1983). The generality of Darwinian principles is a double-edged sword. While generality allows for the wide scope of phenomenon that can be understood in Darwinian terms, it also creates confusion and disagreements over the units of evolution and levels of selection. Life consists of hierarchically nested units (e.g., cells, organisms, social groups), so biologists must determine at which level(s) natural selection is applicable. Further, if evolution by natural selection is occurring at more than one level, the question of the relationship

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between fitness and traits at the different levels must be addressed. Interest in these and related issues has produced a body of literature replete with coined phrases and nuances (e.g., unit of selection versus unit of evolution; p. 13).[1] Controversy concerning levels of selection was central in the turbulent rise of sociobiology as a distinct field (Wilson and Wilson 2007) and has been dismissed by some as a storm in a teacup [p. 240, Waddington as quoted by Maynard Smith (1976)]. Okasha's book *Evolution and the Levels of Selection* (Okasha 2006) shows that the latter is far from the case and that levels-of-selection questions are fundamental problems in biology. Okasha puts the conceptual issues surrounding multilevel selection (MLS) in a coherent framework which serves to highlight the resolution of potential points of confusion (e.g., distinct types of multilevel selection) as well as areas that have great promise for future exploration (e.g., the evolution of life's hierarchical levels). The book is an invaluable resource for anyone interested in evolution, and it is the best book available on the conceptual issues involving MLS. All students and workers interested in multilevel selection should read and study this book.

Okasha critically examines the arguments and implicit assumptions found in the biological literature on multilevel selection. When key assumptions of models or arguments are implicit, productive debate can be slowed by confusion over the assumptions. Okasha's book makes explicit the key points in multilevel selection debates and allows for discussions to be reframed in common terms. Empirical and theoretical biological work could progress more easily by making use of this cohesive conceptual footing.

Evolution and the Levels of Selection has eight chapters. The first four develop a framework for understanding natural selection, and the last four deal with specific topics related to multilevel selection. Chapter 1, "Natural Selection in the Abstract" introduces the concept of natural selection, emphasizing that the abstract conditions necessary for natural selection set the stage for difficulties in understanding natural selection in a biological hierarchy. Okasha contrasts Lewontin (1970) and Maynard Smith's (1987) abstract characterizations of Darwinian principles with those of Dawkins (1976, 1982) and Hull (1981). Okasha favors Lewontin and Maynard Smith's formulations as a starting point because Dawkins and Hull's formulations, being based on "interactors" and "replicators", conflate a product of natural selection with a prerequisite for its occurrence.[2] Okasha introduces the Price equation (Price 1970, 1972), which describes evolutionary change in terms of character-fitness covariance. The Price equation is significant because of its influence on the thinking of many evolutionary biologists concerned with MLS, notably Hamilton (1975). Okasha points out that the correct causal decomposition of the right-hand-side of the Price equation into terms indicating "change due to selection" and "transmission bias" is not commonly seen in the literature. He concludes by positing that the Price equation confirms that Lewontin's conditions

[1] Page references are to *Evolution and the Levels of Selection* unless otherwise stated. We are grateful to Matthew Herron and Aurora Nedelcu for discussions of the ideas in this essay.

[2] Okasha also notes that Lewontin and Maynard Smith treat reproduction as a prerequisite of natural selection, not a product of it. [For a fuller description of the issues implicit in this view, see Okasha (2006), p. 14 and Griesemer (2000)].

are an "extremely good approximation" of the conditions under which evolution by natural selection occurs (p. 37).

Chapter 2, "Selection at Multiple Levels: Concepts and Methods", further develops a framework for understanding natural selection in a hierarchical system. *Evolution and the Levels of Selection* is full of figures of small circles (particles) within larger circles (collectives), representing entities at two different and arbitrary levels of selection. The images illustrate specific points and remind the reader of the generality of the concepts discussed; they also raise the question of what the larger circle means. That is, when should lower-level particles be considered to be part of a hierarchically nested group? Okasha addresses various criteria that have been proposed to answer this question, including among-particle interaction, physical connectedness, and common descent. Okasha uses McShea's approach (McShea 1996, 1998, 2001a, b), in which interaction among parts is the key criterion, and concludes that this "interactionist" interpretation is largely satisfactory. Okasha then discusses the extension of Lewontin's conditions to multiple levels and introduces two distinctions that reemerge throughout the remainder of the book: aggregate versus emergent characters and MLS1 versus MLS2. Aggregate collective characters are a simple function of particle characters (e.g., average gene frequency), while emergent collective characters (e.g., number of cell divisions before germ-line sequestration) are not. The distinction between two types of multilevel selection (MLS1 and MLS2), discussed by authors including Damuth and Heisler (1988), is an important one. In MLS1, particles (i.e., lower-level units) are the focal units (i.e., the units being tracked from generation to generation) while in MLS2 both particles and collectives (i.e., higher-level units) are focal units. Okasha argues that MLS1 and MLS2 are distinct processes that can occur in nature, so a conventionalist interpretation of the distinction should be avoided. Okasha shows that both MLS1 and MLS2 can be described using the Price equation.

Chapter 3, "Causality and Multi-Level Selection", addresses the issues surrounding natural selection as a cause of evolutionary change in a multi-level context. In particular, Okasha emphasizes that character-fitness covariances at a particular level could be the result of selection at a different level, a phenomenon he labels "cross-level by-products." Okasha proposes that the formulation of the levels of selection questions in terms of cross-level byproducts is "particularly sharp" (p. 78) and desirable for several reasons. The concept of cross-level byproducts provides a common framework for understanding specific debates and for approaching various proposals for identification of the "real" level(s) of selection. The concept of cross-level byproducts is a major focus of *Evolution and the Levels of Selection*, and Okasha uses it repeatedly to make sense of subtle debates in the MLS literature. Okasha also introduces contextual analysis, a statistical approach which decomposes change in average particle character value into two terms, one reflecting selection on the particle character and one reflecting selection on the collective character. Contextual analysis can be applied to cases of MLS1 or MLS2, though each demands different considerations, which Okasha addresses. Contextual analysis constitutes an alternative to the more commonly used Price equation approach. Although neither approach appears to fully capture the intuitive requirements for genuine collective-level selection in all cases, Okasha argues that

contextual analysis is usually preferable over the Price approach because it accounts for cross-level byproducts.

Chapter 4, "Philosophical Issues in the Levels-of-Selection Debate", draws on the framework developed in the previous three chapters to address criteria that have been proposed by others for identifying the level(s) of selection. Okasha first argues that the distinction between aggregate and emergent characters is not of fundamental importance for levels of selection debates. He next discusses the additivity of collective fitness (with respect to particle composition), a property with debated relevance to levels-of-selection questions (Lloyd 1988; Sober and Wilson 1994; Wimsatt 1980). Okasha also discusses the Dalmuth/Heisler (Damuth and Heisler 1988; Heisler and Damuth 1987) idea that an emergent *relationship* between a character and fitness is a crucial indicator of a higher level of selection. Okasha sees this idea as highly relevant to levels of selection questions because it serves to identify particle → collective cross-level byproducts. Okasha introduces screening off, a way of analyzing causality in terms of probability that Brandon (Brandon 1982, 1988, 1990; Brandon et al. 1994; Brandon and Carson 1996) has argued is critical for questions of multilevel selection. Okasha points out where both Brandon and Brandon's critics (Sober and Wilson 1994) have gone wrong in understanding the relevance of screening off, which Okasha argues can address cross-level byproducts, the critical concept for identifying levels of selection. Next in Chapter 4, Okasha turns to issues of realism versus pluralism in multilevel selection. He proposes that realism should be the default position and concludes that pluralism (i.e., the claim that there is not a uniquely correct answer to the question of at what level(s) selection is acting) is potentially defendable only under a narrow set of conditions. Finally, Okasha addresses the issue of "reductionism" in multilevel selection, arguing that three distinct ideas are relevant. Okasha equates the question of whether change at a higher level is "reducible" to selection at a lower level with the question of whether a lower → higher cross-level byproduct is responsible for change at the higher level. Interestingly, Okasha draws an explicit contrast between this use of "reduction" and "reductionism" as an explanatory strategy general to all of science. Although this point seems simple, lack of appreciation of the distinct meanings of "reduction" seems to have contributed to several multilevel selection debates (e.g., the debate over the relevance of screening off).

Chapter 5, "The Gene's-Eye View and its Discontents", discusses a case of multilevel selection debate that is intimately tied to issues of reductionism. Okasha traces the origins of "gene's-eye" thinking to Fisher (1930) and Hamilton (1963) and contrasts the process of genic (i.e., gene-level) selection and the gene's-eye perspective. Genic selection is a distinct (and empirically testable) level of selection, while the gene's-eye perspective is a heuristic for thinking about the effects of multilevel selection at one level (that of the gene). Okasha discusses the application of multilevel selection theory to selfish genetic elements, genes that are favored by genic selection but opposed by organismic selection. Finally, Okasha discusses criticisms of the gene's-eye perspective and points out that some of these become unimportant if the distinction between genic selection and the gene's-eye perspective is kept in mind. Okasha claims that the remaining objections apply

mostly to cases in which the genotype–phenotype map is complex, so the gene's-eye perspective is least valuable in these cases.

Chapter 6, "The Group Selection Controversy", covers the historical roots and the present state of debates concerning group selection, where "group" refers to a set of multicellular organisms. Although the conceptual issues concerning multilevel selection could apply to any hierarchal level, the group level has been the focus of heated debate and swings in consensus opinion. Okasha traces the idea of group selection from Darwin's writings, which attribute sterile insect workers to group selection (Darwin 1859), to a period in the 1960s and 1970s in which group selection was largely dismissed as an unimportant mechanism, to the more recent "neo-group selectionist revival". Okasha's exploration of the group selection controversy is illuminating because he uses the concepts explored in previous chapters to expose the heart of current and past debates. For example, he applies ideas concerning group heritability and the MLS1/MLS2 distinction to disentangle key points in a trait-group model debate between Maynard Smith (1987) and Sober (1987) and Sober and Wilson (1998). Okasha concludes that the debate was misplaced and reflected a failure on the part of both parties to appreciate the type of group heritability that is relevant to trait group models. Okasha next describes what Sober and Wilson (1998) call the "averaging fallacy". This fallacy is committed when one calculates the population-wide fitness of a type of individual by averaging across groups and then one claims that types of individuals with a higher population-wide fitness spread by individual selection. Okasha largely agrees with Sober and Wilson (1998); he concurs that this type of claim is indeed fallacious and that particular instances of it are common. Finally, Okasha turns to work by Nunney (1985a, b, 2000) concerning the distinction between strong and weak altruism and the idea that group selection requires assortive grouping. Strong altruism is a behavior that confers a cost on the actor and a benefit on others, while weak altruism is a behavior that confers a benefit on the actor and a larger benefit on others. Nunney argues, in contrast to Sober and Wilson (2002), that weak altruism is really selfish and cannot evolve by group selection. This conclusion as well as the idea that group selection requires assortive grouping hinges on Nunney's use of the neighbor approach. The neighbor approach is a variation on the contextual approach in which evolutionary change is decomposed into an effect from an individual's own character and the characters of its neighbors (i.e., its group, excluding itself). Okasha concludes that Nunney is "partially right" and advocates applying the neighbor or contextual partition on a case-by-case basis. Okasha posits that the neighbor approach is most relevant when groups are not very cohesive.

Chapter 7, "Species Selection, Clade Selection, and Macroevolution", addresses the issue of distinguishing species selection from differential speciation/extinction caused by lower-level processes. In this chapter, now familiar multilevel selection concepts such as emergent characters and "causation from below" are applied to another, higher, level of organization. In spite of the abstract continuity between genic selection, organismal selection, group selection, and species selection, this chapter highlights some novel issues, notably the relationship between a level of selection and individuality. Okasha notes that historically and conceptually, species selection is linked to the "individuality

thesis" of Ghiselin (1974) and Hull (1978). While species lack the functional organization often associated with individuals at other hierarchical levels, the idea that species are individuals (in the sense that they are entities with life cycles) is necessary for them to be considered a potential level of selection. In this chapter, Okasha also agues that clades cannot evolve by cumulative selection because they do not reproduce (i.e., they are monophyletic).

Chapter 8, "Levels of Selection and the Major Evolutionary Transitions", examines the role of multilevel selection in understanding the origin of life's hierarchy. The study of biological evolution concerns the *history* of processes that have produced the complexities of the living world, yet Okasha points out that the debates concerning multilevel selection have, until recently, taken a largely synchronic perspective. That is, multilevel selection has traditionally been discussed in the context of already evolved nested hierarchies (e.g., multicellular organisms or groups of social animals). Of course, life's hierarchically nested units have not always existed, and the challenge of how to account for the emergence of new levels of complexity was posed in terms of the evolution of individuality by Buss (1987) and in terms of "major transitions" by Maynard Smith (1988, 1991) and Maynard Smith and Szathmáry (1995). Major transitions can be understood as evolutionary transitions in individuality (ETIs) because they are characterized by populations of entities (i.e., individuals) evolving in such a way that they lose their autonomy and individuality and become part of a new kind of individual.

Okasha points out that conceptual issues concerning multilevel selection take on new significance and offer new insights when the levels of selection are viewed diachronically. For example, MLS1 (in which the focal level is the "particle") and MLS2 (in which the "collective" and "particle" are focal levels) can be thought of as the beginning (MLS1) and end points (MLS2) of major evolutionary transitions. Okasha labels these beginning and end points Stages 1 and 3 (respectively) and proposes a middle stage (Stage 2) in which "collective fitness is not *defined* as average particle fitness but is *proportional* to average particle fitness" (Okasha's Table 8.1, p. 238, emphasis in original). Additionally, the diachronic perspective highlights the expectation of borderline cases, instances in which entities possess some but not all elements of MLS2 and true individuality.

Using Okasha's three stages, we now discuss the evolution of multicellularity in the volvocine green algae (Chlorophyta), a model system for the evolutionary transition to multicellularity. The volvocine green algae are bi-flagellated, haploid, facultatively sexual eukaryotes found in soil, lakes, and ephemeral pools. The group contains unicellular members (*Chlamydomonas* and *Vitreochlamys*) as well as colonial (i.e., multicellular) relatives with a range of morphological and developmental complexity (Fig. 1). For example, *Basichlamys* and *Tetrabaena* have few features that would distinguish them from a small clump of *Chlamydomanas*-like cells. Species consisting of 8–32 cells have body forms such as a flat or slightly curved single layer of cells (*Gonium*; Fig. 1b), a double layer of cells (*Platydorina*), or spheroidal colonies (*Pandorina*, *Volvulina*, *Eudorina* (Fig. 1d) and *Yamagishiella*). In these species, functional integration of cells is often limited to a few characters. By contrast, the largest forms (various *Volvox* species; Fig. 1f) consist of tens of thousands of cells arranged in a spheroid with clear cellular differentiation

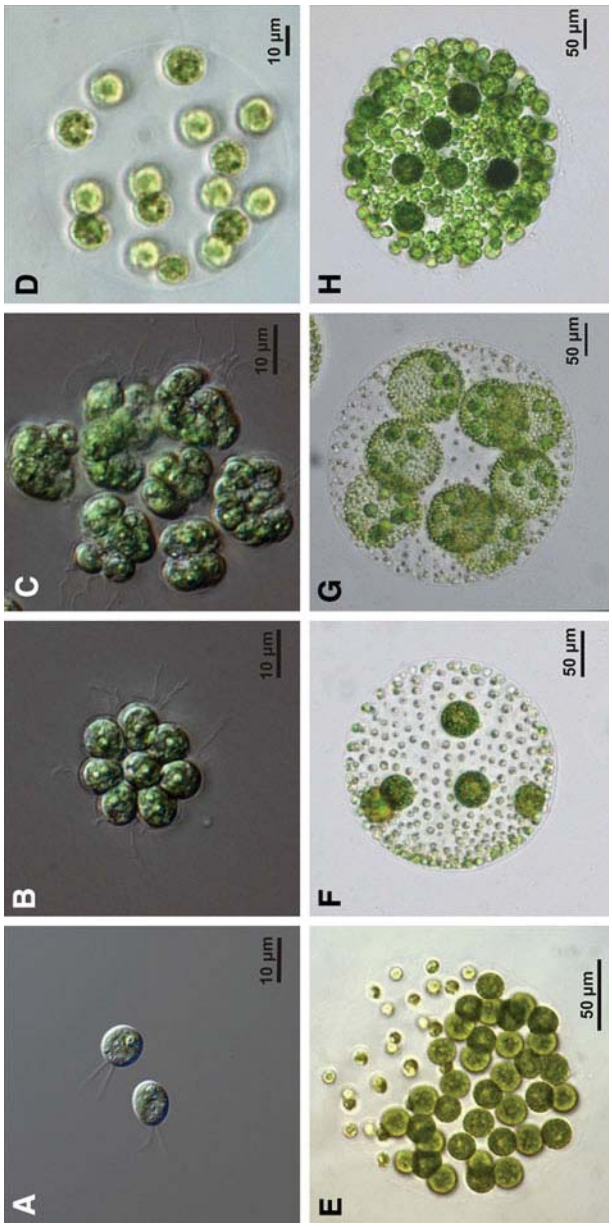

Fig. 1 Body forms and reproduction in select volvocine algae species. **a** *Chlamydomonas reinhardtii* is a unicellular organism with two flagella and is thought to be similar to the unicellular ancestor of the group. **b** *Gonium pectorale* is a colonial species with a relatively simple body form and no germ-soma differentiation. Each cell in these colonies has two flagella, which are oriented so as to allow motility of the colony. **c** *G. pectorale* reproducing and showing eight daughter colonies from an eight-celled parental colony. In *G. pectorale* as well as other volvocine colonies lacking cellular differentiation, each cell produces a colony of the next generation. As discussed in the text, cell fitness and colony fitness are tightly linked for these species. **d** *Eudorina elegans* has a spherical form with undifferentiated cells and (unlike *Gonium* species) an extracellular matrix (ECM) that contributes substantially to the body size and form. Photo courtesy of Matthew Herron. **e** *Pleodorina starrii* colonies consist of ECM with two cell types arranged in a spheroid with larger germ cells and smaller somatic cells at the anterior of the colony (*top*). Photo courtesy of Matthew Herron. **f** *Volvox carteri* (juvenile) colonies also have larger germ and smaller somatic cells connected by an ECM. **g** *V. carteri* (mature adult) in which germ cells have developed into daughter colonies, seen here inside the parental colony. **h** *V. carteri RegA*$^{-}$ colonies with a mutated *RegA* gene in which somatic cells de-differentiate and produce daughter colonies. Mutant strain courtesy of Aurora Nedelcu

into two cell types. Tiny somatic cells contribute to taxis, while much larger reproductive cells produce the next generation of colonies. In these species, cells have specialized in the two basic components of colony fitness, viability and fecundity, so there is little ambiguity about understanding the colony as a level of selection and a full individual.

Part of the appeal of studying the evolutionary transition from unicellular to multicellular organisms in the volvocine algae is the existence of so many living species characterized by different levels of integration among cells. Thus, small evolutionary steps bridging the seemingly wide gap between one level of complexity (unicellular organisms) and another (multicellular organisms) have been proposed for this group (Kirk 2005). Herron and Michod (2008) and Herron et al. (2009) addressed the evolutionary history of these steps through phylogenetic ancestral character state reconstruction, showing that these traits did not arise in a simple, progressive sequence (i.e., some traits arose or were reversed multiple times). Although the history of the steps leading to volvocine multicellularity is complicated and distinct for many lineages, Herron and Michod (2008) identified patterns in the order of emergence of traits that could reflect the cycles of cooperation, conflict, and conflict mediation among cells predicted by multilevel selection theory.

It would be an entire research program to discover and describe the correspondences between all the terms and issues in MLS theory and a diverse group of organisms like the volvocine algae. Here, we revisit the pattern of evolution of volvocine traits described by Herron and Michod (2008) and Herron et al. (2009) and discuss Okasha's concept of a three-stage transition with reference to this group. Okasha describes the generalized progression of an ETI, making reference to the applicable type of MLS (one or two) and to the relationship between collective and particle fitness during the transition. He describes the initial stage of a major transition as characterized by the spread of cooperation among lower-level entities before cohesive collectives have evolved (pp. 229–231). Examples of how this initial stage could be relevant to ETIs include the spread of cooperation or altruism among multicellular animals prior to a transition to eusociality or the spread of cooperation in hypercycles of RNA molecules prior to the transition to gene networks. In Okasha's description and in these examples, MLS1 is the relevant type of framework. In distinguishing between MLS1 and MLS2, the key issue is to identify the "focal level" or what Sober (1984) called the "benchmark of selection" (as quoted in Okasha 2006, p. 56). In MLS1, the *explanadum* (in these examples, "particle cooperation") is at the lower level, so the lower level is the focal level. The distinction follows the two ways in which collective fitness can be defined. In MLS1, the fittest collectives are those that contribute the most particles to subsequent generations. That is, collective fitness is what Okasha calls collective fitness_1 and is defined as the average or total fitness of the collective's constituent particles (pp. 53–59 and p. 238, Okasha Table 8.1).

Applying the idea of Stage 1 in an ETI to the volvocine transition to multicellularity is not as straightforward as it might first appear. Herron and Michod (2008) suggest that the initial cooperative trait to spread among a common ancestor of multicellular volvocine algae was the transformation of the outer cell wall into an

extracellular matrix (ECM).[3] Multicellular volvocine algae all have ECM, which consists of glycoproteins and is homologous with the outer layer of the *C. reinhardtii* cell wall. In extant multicellular volvocine algae, the ECM functions to keep cells in a colony together and also acts as a kind of commons for the storage of temporarily abundant nutrients. Herron and Michod (2008) interpret the initial evolution of the ECM as cooperative because individual cells could contribute to a common resource which was beneficial to all cells in the group.

Envisioning ECM as the initial cooperative trait to spread among volvocine cells is dissimilar to the other examples of Okasha's Stage 1 because of the way in which volvocine algae reproduce. In colonial volvocine algae, each reproductive cell (or every cell in undifferentiated species in which all cells reproduce) gives rise to a new individual colony of the next generation (Fig. 1c and g). Cells typically grow to many-fold their original size, then divide rapidly a number of times to produce a daughter colony. The resulting daughter cells remain together with each other but separate from the similarly-produced daughter colonies of other parental cells. So, for e.g., in an eight-celled *Gonium* colony, each cell would typically grow eight-fold and produce an eight-celled daughter colony; a single *Gonium* colony would produce eight daughter colonies (Fig. 1c). This mode of reproduction is known as autocolony. Group formation by failure of daughter cells to separate can even be seen in single-celled volvocine algae. The single-celled *Chamydomonas reinhardtii* is known to form clumps of daughter cells that fail to separate (so-called palmelloid colonies) under some laboratory conditions (Iwasa and Murakami 1968, 1969). A mutation in a gene involved in the posttranslational modification of glycoproteins, the primary component of *C. reinhardtii* cell walls, can cause *C. reinhardtii* palmelloid colonies to form (Vallon and Wollman 1995), but the generality of this mechanism of palmelloid formation is not known. Palmelloid colonies are not just a laboratory curiosity; they have been shown to form in response to predation by rotifers (Lurling and Beekman 2006). Thus, palmelloid formation may be adaptive under some circumstances, allowing for *C. reinhardtii* cells to escape from predators only capable of eating cells or clumps of cells below a particular size.

The hypothesis that ECM production was the initial means of among-cell cooperation and the observation of cell wall-mediated clumping of single-celled volvocine algae complicates interpreting the volvocine transition to multicellularity as beginning with Okasha's Stage 1. It seems possible that among-cell cooperation in the ancestors of multicellular volvocine algae immediately produced a level of group cohesion and reproduction that does not fit with other examples of "Stage 1" (such as hypercycles, social insects, slime molds, etc.). While the *explanadum* in the volvocine case would still be cell (lower)-level cooperation, the immediate emergence of a cohesive colony (higher unit) capable of reproducing more colonies, makes collective fitness$_2$ (defined as expected number of offspring collectives), and therefore MLS2 and Okasha's later stages, seem immediately applicable. This may be a general feature of other multicellularity transitions, because the autocolony

[3] Herron and Michod's (2008) analysis indicates that the presence of ECM as well as genetic modulation of cell number are likely ancestral to all colonial species. The order of emergence of these two traits could not be resolved, but they hypothesize that presence of ECM preceded genetic modulation of cell number.

mode of group formation is an example of what Bonner (2000) postulated was the most straightforward and general way of cells to make multicellular groups: failure of daughter cells to separate and go their own way following cell divisions.

The above line of thought would indicate that Stage 1, as described by Okasha, is not applicable to the evolution of multicellularity in the volvocine algae. However, one could also consider other aspects of Stage 1 and conclude it is applicable not only to the ancestors in which cell–cell cooperation first emerged, but also to some extant colonial forms. In Stage 1, "collective fitness is defined as average particle fitness" (p. 238, Okasha Table 8.1); the fittest collectives contribute the most particles to subsequent generations. For the purposes of discussion, we will take fitness as expected reproductive success, a definition applicable to organisms with discrete generations (as in these algae) when generation time is constant (probably not applicable to these algae). Although there have not been systematic studies of within-colony cell mortality in volvocine colonies, cell survival is directly tied to colony survival. Cell reproductive success is just the number of cells in a colony, and the reproductive success of a colony is also the number of cells in the colony. Consequently, colony fitness is equal to average cell fitness for all non-differentiated volvocine algae (species with no germ-soma differentiation).

For example, in eight-celled *Gonium* colonies, a single cell produces eight daughter cells kept together in a single eight-celled colony. A single *Gonium* colony produces eight daughter colonies (Fig. 1c). So the colony-level reproductive success equals eight, which is the cell level reproductive success. Equating cell survival and colony survival, colony fitness is given to be average cell fitness.

Several caveats apply to the view that *Gonium* and other undifferentiated volvocine colonies are examples of Stage 1. First, this view is based on a specific definition of fitness, expected reproductive success, which requires the assumption of equal generation times among individuals. Relaxing this assumption and operationalizing fitness differently (e.g., with the Malthusian parameter) could lead to different conclusions about the relationship between cell and colony fitness. Although Okasha (pp. 49–56) discusses some points of confusion in applying the concept of fitness to both particles and collectives, he does not address the implications of accounting for among-individual variation in generation time at one or both levels. Indeed, for a philosophical book about natural selection, there is rather little discussion about how to define fitness. A second caveat is that viewing *Gonium* as Stage 1 seems to rest on the assumption that within-group variation in cell fitness is absent. That is, in our example of an eight-celled *Gonium*, each cell within a particular colony has the same fate: either not surviving to reproduction or surviving and producing eight daughter cells. This assumption is unsatisfying because there is little empirical evidence to support or disprove this scenario (but see Smith (1944) for a description of variation within *Volvox* colonies). It is also unsatisfying because accepting this assumption would make *Gonium* properly classified as Stage 1 (because colony fitness would equal average cell fitness), but it would do so by eliminating within-group variation in fitness at the lower level, a hallmark of MLS1 and of Okasha's Stage 1 example of RNA molecule hypercycles. This is also inconsistent with the view that lack of within-colony variation in cell fitness usually results from a colony-level adaptation that promotes colony function

by suppressing conflicts between cells. Clearly colony-level adaptations should not be posited at the *beginning* of a transition to colony-level selection.

Herron and Michod (2008) show that, along with transformation of the cell wall into ECM, genetic control of cell number was among the first distinct traits to arise in colonial volvocine algae. They suggest that genetic control of cell number could be an adaptation to mediate conflict among cells because cells that failed to invest in a group-beneficial trait (i.e., ECM) would be unable to channel the saved energy towards increasing their individual reproduction. If this scenario is correct, then extant colonial volvocine algae (which all have genetic control of cell number) are the product of at least one round of conflict and conflict mediation, so it would be difficult to understand any of them as representative of the initiation of an ETI (i.e., Stage 1).

In Okasha's Stage 2, "collective fitness is not *defined* as average particle fitness, but is *proportional* to average particle fitness" (p. 238). This stage is described largely in relation to a model by Roze and Michod (2001) in which fitness of the cell collective (i.e., higher-level entity) is determined in part by collective functionality and in part by collective cell number (which depends on the reproduction of lower-level cells). That is, the fitness of the collective is not fully de-coupled from the fitness of the constituent cells.

Perhaps the extant colonial volvocine species that lack germ-soma separation (e.g., *Basichlamys*, *Tetrabaena*, *Gonium*, and *Eudorina*) are best understood as examples of Okasha's Stage 2. In these colonies, cell fitness contributes directly to colony fitness. Each cell produces a colony of the next generation. Colonies have emerged as entities in their own right (with life cycles), and colony fitness seems to depend, perhaps to differing extents, on the functionality of the colony. Yet collective fitness is still intimately coupled to cell fitness. As in the Roze and Michod (2001) model, colony (i.e., group, collective) fitness depends both on colony functionality and on cell reproduction.

By contrast, colonies with complete germ-soma cellular differentiation (e.g., *Volvox*) are clearly cases of Okasha's Stage 3. Average particle fitness is zero because both germ and soma cells fail to invest in a major aspect of fitness (reproduction or viability), yet colony fitness can be high. Thus, the fitnesses of the two levels are completely decoupled, and multicellular colonies with germ-soma differentiation can be considered to be true individuals. Interestingly, mutation in a single gene (*regA*) seems to cause *Volvox carteri* colonies to regress back to a Stage 2-like state. In *regA* mutants, somatic cells de-differentiate and produce daughter colonies (Fig. 1h), in essence reclaiming their cell-level fitness (see Kirk (1998) for further information). The ease with which volvocine somatic cells can de-differentiate to produce colonies raises the possibility that the mechanism of fitness decoupling had important consequences for the evolutionary potential of this taxon. In metazoans, somatic cells reproduce, differentiating into an impressive array of types, and many mechanisms of preventing somatic cells from "reclaiming" cell-level fitness (i.e., as in cancer) exist. By contrast, somatic cells in wild-type volvocine algae do not reproduce and are all essentially similar. Could the seemingly crude mechanism of fitness decoupling in volvocine algae (i.e., preventing somatic cells from reproducing at all) have prevented

the further diversification of somatic cell types and body forms (Nedelcu and Michod 2003)?

In summary, all aspects of Okasha's three-stage scheme for evolutionary transitions may not be generally applicable to all ETIs. Stage 1, the spread of cooperation in the absence of cohesive groups, and Stage 2, the transition between MLS1 and MLS2, are particularly difficult to understand as they apply to volvocine algae. As Okasha points out, the diachronic perspective makes the existence of borderline cases, and the difficulty of applying concepts to them, expected. Okasha's three stages are a provocative step towards more conceptual clarity in regard to ETIs. Further exploration of Okasha's three stages and the concepts on which they are based is certainly warranted. Applying these ideas to the volvocine algae, as well as to other multicellular taxa that differ in important respects (e.g., amoebae, fungi, plants, and animals), could produce a deeper and more general understanding of major evolutionary transitions.

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APPENDIX B. GROUP SELECTION AND GROUP ADAPTATION DURING A MAJOR EVOLUTIONARY TRANSITION: INSIGHTS FROM THE EVOLUTION OF MULTICELLULARITY IN THE VOLVOCINE ALGAE

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Abstract

Adaptations can occur at different hierarchical levels (e.g., cells and multicellular organisms), but it can be difficult to identify the level(s) of adaptation in specific cases. A major problem is that selection at a lower level can filter up, creating the illusion of selection at a higher level. We use optimality modeling of the volvocine algae to explore the emergence of genuine group (i.e., colony-level) adaptations. We find that it is helpful to develop an explicit model for what group fitness would be in the absence of group-level relationships between traits and group fitness. We call this "counterfactual fitness," because in many actual cases of interest there are group-level relationships. Once counterfactual fitness is modeled, the difference between effects that filter up and genuine group selection is explicit and so, therefore, is the distinction between apparent and genuine group adaptations. We call the latter group-specific adaptations. Recognizing group-specific adaptations is important because only group-specific adaptations would cause the lower-level units (cells in this case) to be maladapted if they were to leave the group and enter a global cell-level population. Thus, as group-specific adaptations evolve, they create selective pressure for increased cohesiveness and individuality of groups. This work suggests that group-specific adaptations could be present in the simplest, earliest branching colonial volvocine species which do not have distinct specialized cells. The work also makes predictions about the kind of empirical evidence needed to support or refute the hypothesis that a particular trait is a group-level adaptation.

Introduction

The emergence of a new kind of population that evolves by natural selection has been called a major evolutionary transition (Maynard Smith and Szathmáry 1995) or an evolutionary transition in individuality (ETI; e.g., Michod 1996; Michod 1999). During ETIs, groups of individuals evolve into a new kind of individual. Our focus here is on a particular ETI, that of the evolution of multicellularity, in which groups of cells become a new kind of individual: the multicellular organism. We use the evolution of the colonial body forms in the Volvocaceae, a family of green algae, as a case study to inform our understanding of group selection and group adaptation. ETIs challenge us to think carefully about how to generalize fundamental evolutionary biology concepts such as selection and adaptation to cases with emerging hierarchical structure.

In order to explore the emergence of group selection and group adaptation, we develop a model which is inspired by fundamental aspects of volvocine algae biology. "Volvocine" is an informal term referring to the Volvocaceae (a family of all colonial/multicellular species) plus some closely related species. Volvocine algal species range from unicellular to simple colonies of undifferentiated cells to highly integrated colonies with cell-level differentiation. Existing volvocine species span the divide between fully unicellular and fully multicellular (Kirk 2005). The motive of focusing on volvocine algae is not to illuminate volvocine-specific issues. Rather, we think that focusing on a biological system with many intermediate forms makes it easier to approach the abstract and subtle issues raised when generalizing natural selection and adaptation to hierarchically structured populations. We use the terms "cell", and "lower level" interchangeably and likewise for the terms "group", "colony", and "higher level". We generally use "individual" to refer to cells; where "individual" refers to colonies, this is made clear.

The basic challenge is to explain how, during a major evolutionary transition, a kind of entity that did not undergo natural selection before begins to undergo natural selection. Volvocine algae can give us a concrete example of what changes as an evolving population of separate cells becomes an evolving population of cohesive, integrated cell-groups. The end of an ETI is characterized by both natural selection and adaptation shifting to the higher level. A critical aspect of such transitions may be the evolution of traits that limit or suppress lower-level natural selection and adaptation (e.g., Michod 1996; Michod 1999; Michod 2003). Much attention has focused on the potential conflicts between natural selection at the lower and higher-levels. Here, we are more concerned with the path(s) by which the group level of organization can first become established. In volvocine algae, the number of cells in a group was probably quite low when clonally-derived cell-groups first evolved. Therefore, much of the conflict that comes from competing cell lineages within a large organism (or cell-group) was probably not relevant to this early stage. Nonetheless, it is illuminating to question the role of natural selection and adaptation in these nearly-conflict-free, small clonal groups. Assuming there is selective pressure for larger size, just how much group adaptation can become established before the groups become large enough to face the problem of within-group cell lineage competition? Can we apply the distinction between a group adaptation and a group trait that happens to benefit the group? Does the distinction matter for understanding the early stages of an ETI?

We first explore the concept of adaptation in more depth and introduce the idea that groups can have traits that look like group-level adaptations but are not. Because of the close link between adaptations and optimality modeling, we then construct an optimal life history model based on the biology of volvocine algae. Life history traits affect fitness and are subject to selection and constraints. As such, they are fruitfully approached from a design-principles or

optimality perspective (Stearns 1992). In scenario A, we develop a hypothesis based on life history theory of what adaptations might have been like in an ancestral unicellular volvocine species. We then consider a case in which cells are organized into simple colonies but without true group-level effects (scenario B). We address the way that unicellular trait-fitness relationships could filter up the hierarchy, resulting in spurious group-level effects. Finally, in scenario C, we consider a case in which there is a mixture of effects that filter up from below as well as genuine cause-effect relationships between traits and survival or reproduction at the group level. Next, we introduce the Price equation and related approaches to partitioning levels of selection. We propose a method of partitioning trait change by splitting the covariance term in the Price equation into a term that corresponds to group-specific selection and a term that corresponds to global individual selection. The proposed partition is an addition to the traditional Price equation partition, indicating that two hierarchical levels of selection could lead to three distinct categories of natural selection (i.e., two categories even in the absence of within-group change). Finally, we discuss the potential importance of the proposed partition in the context of ETIs.

Adaptation and the fleet deer problem

An adaptation refers to a feature of an individual that has been influenced by past natural selection to be well-suited to meet a selective demand of the environment. Here "environment" simply refers to factors that can be considered to a first approximation to be external and separate from the evolving population itself; examples include weather patterns, predators, etc. Feedback between "environmental" conditions and the evolving population complicate this picture, but we set aside that kind of complication here. Informally, we recognize adaptations as a match between an individual's traits and the specific demands of its environment. Authors have

different ways of making the idea of adaptation more concrete. Van Valen (2009) conceptualizes an adaptation as a feature of an organism that "provides greater fitness than at least some adjacent or nearby parts of the relevant state space, now or in the evolutionary immediate past". Parker and Maynard Smith (1990) argue that optimality modeling is a useful way of constructing and testing hypotheses about the adaptiveness of traits. They also emphasize, however, that optimality modeling does not require the assumption or attempt to prove that traits of organisms are optimal (Parker and Maynard Smith 1990). This is consistent with Van Valen (2009), who writes that an adaptation is simply a feature that enhances fitness. By this view, a trait value need not be optimal even locally to be an adaptation, though some of the nearby (i.e. easily accessible by evolution) trait space must consist of less fit alternatives. There are countless awe-inspiring examples of adaptation; just one example is the way an owl's fringed feathers muffle sound, helping the owl to silently approach its next meal (e.g. Bachmann, Wagner, and Tropea 2012; Chen et al. 2012). Yet even for a single level of organization, the concept of adaptation is not always easy to apply (cf. Stearns 1992, 16–19). Note that the notion of function or purpose often goes hand in hand with the concept of adaptation. One could say that the (primary) function of the fringes on the feathers is sound-muffling. Recognition that a trait is likely to be an adaptation can occur independently of recognition of its function (i.e., what the trait is an adaptation for; Van Valen 2009), though often evidence that a trait is adaptive is stronger when its function is understood. The function of an adaptation seems teleological; for example, an owl develops fringed feathers in apparent anticipation of the need to have sound muffled. Of course, the explanation for this apparent purpose lies in the repetitiveness of life cycles and in the commonalities between the problems encountered by parents and offspring (Michod 1999, 163–164). Also note that the concept of an adaptation as a trait that is well-suited to meet a selective

demand requires some notion of an alternative case (Williams 1966). That is, recognizing an adaptation requires knowledge of what the feature *would* look like if it were not affected by the selective demand under consideration. Whether a feature is considered an adaptation (or not) ultimately depends on the trait's relationship to fitness. Selective demands are specified by the relationship between trait values and fitness. For example, the higher fitness of (otherwise similar) owls with a better ability to muffle flight sounds when catching prey would be a selective demand. In the absence of a history of this kind of trait-fitness relationship, sound-muffling-ability would not be considered an adaptation. Understanding and representing selective demands (trait-fitness relationships) is the essence of optimality modeling. Therefore, the close connection between the concept of an adaptation and the results of an optimality model makes sense.

Paley (1802) famously argued that there cannot be design without designer. In contrast to Paley (1802), we now know that natural selection is the process which explains biological design and adaptation. Nonetheless, it can be illuminating to return to Paley's (1802) argument when parsing out exactly how we now conceive of biological adaptations. Paley (1802) writes: "There cannot be… means suitable to an end, and executing their office in accomplishing that end, without the end ever having been contemplated, or the means accommodated to it." The modern concept of adaptation indeed does entail a means/end distinction. An adaptation is a means of meeting a selective demand. Natural selection is the process that accommodates the means (individual features) to the ends (meeting the environmental challenges). Supposing that the end must have been contemplated is superfluous, but Paley (1802) was on the right track in thinking that the appearance of means/end relationships in organisms (i.e., the existence of features with unambiguous functions) implies a process by which the means were accommodated to the ends.

In tackling the issue of extending the concept of adaptation to a hierarchical setting, we will largely focus on the notion of adaptations as means for meeting (or better meeting) selective demands. We will also focus on natural selection as a process that explains adaptations. That is, if a process is called group-level natural selection, then it should produce group-level adaptations in the absence of complicating factors which also thwart individual-level adaptation (e.g., migration, too low heritability, etc.). Likewise, if a feature is a group-level adaptation, then it must have been produced by group-level natural selection.

A complication to extending the concept of adaptation to the group level was described in a memorable way by Williams (1966). He described a herd of fleet deer that is good at avoiding herd-level mortality due only to the aggregated adaptations of individual deer and not any additional advantage that is specific to the group level. Williams (1966) argued that calling such a group a "fleet herd of deer" would imply that average herd speed had increased due to group-level natural selection and would be misleading. The situation in which the evolution of a higher (colony)-level trait is influenced only by natural selection at the lower (cell)-level and not by natural selection at the higher level has been dubbed a fortuitous benefit (Williams 1966) or cross-level byproduct (Okasha 2006, 78).

Volvocine algae model

In order to model adaptations in volvocine algae, we first operationalize fitness. The concept of fitness has been a major focus of discussion in evolutionary biology, and we do not attempt to review the subject here. We instead aim to isolate the issue of group adaptation by considering cases that share a particular operational meaning of fitness. We assume a growing population (of cells or colonies) with no density- or frequency-dependent fitness effects. In short, we assume conditions that make r, the intrinsic rate of increase, an appropriate way to operationalize fitness

(i.e., an appropriate maximand). Because volvocine algae commonly colonize a habitat, increase in numbers, then make dormant spores (Kirk 1998), basing fitness on r is reasonable and relevant to the algae's natural conditions (see Stearns 1992, 33 for more discussion on fitness). In a population with non-overlapping discrete generations, Malthusian fitness or r can be approximated by (Andrewartha and Birch 1954; Charlesworth 1980; Roff 2001):

$$r = \frac{\ln(\text{fecundity} \times \text{survival})}{\text{generation time}} \quad (1)$$

Fecundity (F) is the number of offspring per generation (conditioned on survival) and survival is the per-generation probability of survival. (See Table 1 for a description of symbols used.) Fecundity times survival is the expected number of offspring per generation. We assume individuals die at a constant rate M and generation time is given by T_t ($\text{survival} = \exp(-MT_t)$). We refer to $\frac{\ln(F)}{T_t}$ and M as fitness components. Substituting for survival, we see that the intrinsic rate of increase (fitness) of the cell population is given by:

$$r = \frac{\ln(F)}{T_t} - M \quad (2)$$

Next, we consider a fundamental cell-level life history trade-off. This trade-off is simplified compared to more realistic biological examples, but is nonetheless motivated from plausible physical constraints. A cell, at any given time, has a fixed energy and resource budget. Gene expression requires energy and resources. Gene products that promote the survival ability of the cell are often not the same as the gene products that promote the growing ability of the cell. Therefore, a cell must "decide" to allocate limited energy or resources to expressing either genes related to defense (i.e., survival) or to expressing genes related to photosynthesis (i.e., growth). The compromise that the cell reaches affects two of the cell-level properties we are concerned with: the instantaneous rate of cell death and the growth rate of the cell. Although this

life history decision is actually a dynamic one, made throughout the lifetime of the cell, we simplify the picture by assuming that this decision is made once at a particular stage in the life cycle. Each kind of cell is characterized by a constant value, g, indicative of the proportion of total energy or resources invested in growth. The value of g is assumed to be determined by the genotype of a cell. As the amount invested in growth (g) increases, both the cell growth rate and the cell instantaneous mortality rate also increase.

Scenario A: unicellular population

We now develop a specific version of the model based on assumptions about an entirely unicellular population of algae. We call this "scenario A" and use the superscript A to indicate scenario A. We use the subscript L to indicate reference to the lower (cell) level and H to indicate reference to the higher (colony) level. Scenario A concerns a population of unicellular algae, assumed to be similar to the unicellular ancestor of the colonial species considered in later scenarios. We have in mind something loosely like *Chlamydomonas reinhardtii*, an extant unicellular biflagellated green alga. This species is facultatively sexual, though we focus on the asexual portion of the life cycle here. The asexual cycle is palintomic (long period of growth interspersed with a series of rapid divisions; Figure 1).

In scenario A cell-level fitness is given by:

$$r_L^A = \frac{\ln\left(\frac{c + k_g g t_s}{c}\right)}{T_t} - k_m g \tag{3}$$

See Appendix 1 for a description of scenario A cell-level fitness. Three variables (k_g, k_m, and t_s) are assumed to be set by the environment. Two variables (c and T_t) are assumed to be fixed for all cells for the conditions under consideration. Only the value of g (allocation to growth) is assumed to be set by the genotype. An example of the relationship between g and fitness is

shown in Figure 2. Depending on the other parameter values (i.e., on the environment), selection on g could be directional (with full allocation to growth favored) or stabilizing (with intermediate values of g favored). The main point with regard to adaptation is simply that in a given environment, r_L^A will be maximal for some value of cell investment in growth (g). The elements relevant to cell-level adaptation are clearly specified for this scenario. The trait that can vary among different kinds of cells is g (proportion of energy or resources invested in cell growth). The maximand is cell Malthusian fitness, r_L^A. Finally, the constraint set consists of the ways in which g affects fitness.

Scenario B: groups exist but do not affect fitness

Hierarchical levels of organization raise the possibility of groups having features that promote group survival or reproduction as a side-effect of natural selection at the lower (individual) level. Scenario B is meant to capture this idea by proposing a case in which volvocine algae colonies exist, but colony membership does not affect the previously-specified relationships between cell traits and cell fitness components. That is, scenario B is modeled to represent a case of "cross-level byproduct" (Okasha 2006, 78) or a "fortuitous" group benefit (Williams 1966). A colony life cycle could emerge from a mutation that leads to some sort of adhesive extracellular material that is strong enough to temporarily hold offspring cells together, as depicted in Figure 1. Thus, groups form when a single large cell undergoes a rapid series of divisions and the mitotic products do not immediately separate. The cells stay together as a colony for a while, and each cell within the colony increases in size. Groups (colonies) dissolve when all of the cells of a colony finally separate from each other. Group dissolution occurs after cells have grown for a time, but before any cell divisions have occurred. That is, all groups dissolve completely and

there are never groups within groups. Each, now independent, large cell eventually undergoes a rapid series of divisions, completing the cycle.

For scenario B, we have in mind something like the simplest colonial volvocine algae species, such as *Basichlamys sacculiferum*. Cells in a *B. sacculiferum* colony, at first glance, do not appear or behave much differently compared to the presumed state of their unicellular ancestors. These colonies are poorly integrated. Cells do not orient their flagella according to their location in the colony, nor do they specialize in either reproduction or somatic activities, as in some of their larger and more complex colonial relatives. There is no cohesive boundary surrounding the entire colony. Cells are not even attached to each other, but rather are each attached to cell wall material remaining from the parent (Iyengar and Desikachary 1981; Ettl 1983; Nozaki, Itoh, Watanabe, and Kuroiwa 2011). This is not to say that scenario B—in which we assume that the colony has no relevant effects—is actualized in *B. sacculiferum*. However, if there were a scenario B case, it might look something like *B. sacculiferum*.

The ways that g affects cell growth rate and cell mortality rate remain unchanged compared to the unicellular scenario A. Note that because colonies are assumed to have no effect in this scenario, the death of an entire colony by some factor that affects the colony as a whole is not possible. Cells within a colony are subject to mortality independently of one another. If we took the perspective of an individual cell within a scenario B colony, the functional dependence of its cell-level fitness on g would be the same as was specified in scenario A. In scenario B, cell-groups exist in the sense of there being spatio-temporally discrete aggregations of cells; however, groups in the sense of a trait-group (Wilson 1975) do not exist. A trait group is a group that is defined by the existence of fitness-affecting interactions (which can be independent of spatio-temporal proximity).

The only difference between scenario A and B is that, given scenario B, it is possible to write down colony-level fitness components. For any cell trait value (g), the value of r_H^B (intrinsic rate of increase of the population of colonies) is identical to the value of r_L^B (Appendix 1, A10-A21). The value of r_H equals the value of r_L within all scenarios because the groups are assumed to be completely homogenous; cell and colony level fitness within any scenario are mathematically equivalent ways of bookkeeping.

Scenario C: groups exist and affect fitness

Now we consider a case which is similar to scenario B, except that the colony itself has an assumed effect on the trait-fitness relationships. Group formation and dissolution occurs as described for scenario B. Cells within a colony are still assumed to be capable of dying independently of one another, as in scenario B. However, in scenario C, the number of cells per colony affects the mortality risk of the cell. For example, one could imagine a predator that locates its prey in a size-dependent manner (e.g., more easily spotting larger colonies) but can only attack one cell (i.e., one individual within the group) rather than consuming the group as whole. The extent to which cells within volvocine algal colonies actually die independently of one another—and how colony cell number may influence this process—is not yet known in practice. Nevertheless, it is reasonable to assume that colony size would affect cell mortality risk. The advantage of this as a starting point is that it constitutes a small modification from the cross-level byproduct case (scenario B). This framework could be extended to also include the risk of mortality to whole colonies (rather than independent cells). Other colony-level effects, such as an influence of colony cell number on cell growth rate are also likely to occur. For simplicity, we consider only an effect of colony cell number on cell mortality rate here.

In contrast to scenarios A and B (where cell mortality was given by $k_m g$), we now define the cell instantaneous rate of mortality for scenario C to be:

$$M_L^C = \frac{k_m g(\alpha + \beta)}{\alpha\left(\frac{c + k_g g t_s}{c}\right) + \beta} \tag{4}$$

where α and β are positive constants that determine the reduction in cell mortality rate that occurs as number of cells per colony at birth $\left(\frac{c+k_g g t_s}{c}\right)$ increases. The rest of the components of fitness remain unchanged from Scenario B, giving the following for Scenario C cell-level fitness:

$$r_L^C = \frac{\ln\left(\frac{c + k_g g t_s}{c}\right)}{T_t} - \frac{k_m g(\alpha + \beta)}{\alpha\left(\frac{c + k_g g t_s}{c}\right) + \beta} \tag{5}$$

Note that we have two different fitness functions. First, the cell-level fitness in scenario A equals the cell-level fitness in scenario B as well as the colony-level fitness in scenario B (Equation 3). Second, the cell-level and colony-level fitness in scenario C (Equation 5) are equivalent to each other (Appendix 1, A23–A27) but different from the scenario A and B fitness function. Finally, consider two subsets of scenario C. In scenario C1, the effect of colony cell number on cell survival is extremely minor ($\alpha = 0.01$ and $\beta = 50$). In scenario C2, the effect of colony cell number on cell survival is more substantial ($\alpha = 0.01$ and $\beta = 1$). C1 and C2 are distinguished only by different values for β, a constant related to the group-level trait-fitness relationship. Whereas the dependence of fitness on g is almost identical to the cross-level byproduct case in scenario C1, the dependence of fitness on g is very different from the cross-level byproduct case in scenario C2 (Figure 2).

Natural selection and levels of selection

To explore how the volvocine algae model can inform our understanding of natural selection and adaptation, we first turn to some standard treatments of natural selection as an abstract process. A common approach to specifying what is meant by natural selection (at a single-level) is to say that natural selection is the process that occurs when particular pre-conditions are met (reviewed in Godfrey-Smith 2007). Okasha (2006, 18) summarizes this approach by stating: "a population of entities evolves by natural selection where heritable differences between the entities lead to differences in their reproductive output; reproduction is understood as giving rise to an offspring entity that occupies the same hierarchical level as the parent, unless otherwise stated." This kind of approach is not universally endorsed (e.g., Bouchard 2011; Earnshaw-Whyte 2012). Nevertheless, understanding natural selection as change in the prevalence of a trait type due to heritable differences in the trait combined with effect(s) of the trait on survival and reproduction captures the essence of the process described by Darwin (1859).

The Price equation (Price 1970; 1972) is one useful way to formalize what is meant by natural selection in the abstract and can be thought of as a formalization of the conditions approach (Okasha 2006). The Price equation is commonly presented as

$$\Delta\bar{z} = \mathrm{Cov}(w_i, z_i) + \mathrm{E}(w_i \Delta z_i). \quad (6)$$

The value of the measurement of a trait on parental type *i* is given by z_i, and z_i' is the average value of the trait in the descendants of parents with the index i. The change in trait value between a parental type and its descendants is given by $\Delta z_i = z_i' - z_i$. Relative fitness (w_i) of an *i*-type is the *i*-type's number of offspring divided by the average number of offspring produced by members of the parental population. For an example of a derivation of the Price equation see Frank 2012. The partition in the right-hand-side of Equation 6 is often equated with change due

to selection (first term) and change due to transmission (second term). Alternative partitions of change associated with natural selection versus transmission have been discussed. For discussion see Frank (2012) and Okasha (2006). It is important to note that the Price approach to partitioning trait change associated with selection versus trait change associated with trait transmission has implications when multi- instead of single-level scenarios are considered (Price 1972; Hamilton 1975; Wade 1985). This is because transmission bias at one level can be the result of natural selection at a lower (within-group) level. Segregation distortion is a clear example of this (Lyttle 1991; Van Boven and Weissing 1999). So the task of separating change due to transmission bias is intimately related to the task of separating change due to selection at different levels. We set aside this kind of multi-level issue and consider the case when transmission bias is completely absent ($\Delta z_i = 0$ for all i).

We consider two general categories of question related to natural selection first at a single level and later at the group level:

i) Can the change of some particular trait in a population be attributed (or partially attributed) to natural selection? Put another way: is there selection *of* this trait?

ii) Do differences in the trait in question cause fitness differences? Put another way: is there selection *for* this trait?

Sober (1993, 97–102) emphasized the distinction between "selection of" versus "selection for". The distinction is related to the familiar point that a correlation can occur without a direct causal link between the two correlated items. The distinction between a cause-effect relationship and a mere correlation (without a direct causal link) is especially important in the context of natural selection. Natural selection was initially conceived of as an explanation for the appearance of design in the biological world (i.e., for adaptations). Natural selection as an explanation of

changes in population trait values that lead to the appearance of design (adaptive changes) hinges on a causal, not just statistical, connection between a trait value and the representation of an individual's descendants in the future population. For example, consider a case in which average body size increases in a population because larger body size is correlated with larger brain size and larger brain size causes better reproductive success. One would not want to call the larger body size that evolved an adaptation. The fact that larger body size is not an adaptation could be demonstrated by experimental manipulation in which brain size was uncoupled from body size. In this situation, increasing an individual's body size only would not lead to better performance at any activity related to survival or reproductive success; larger body size would fail the design-principles criteria of an adaptation. There would not be a fit between body size and environment that would be observable from a design-principles perspective. Nevertheless, it would be accurate to say that there was selection *of* body size when body and brain size were correlated. Adaptive evolution of a trait hinges on there being selection *for* that trait, not simply selection of that trait.

With respect to the Price equation term typically thought to correspond to single-level natural selection (first term in right-hand side of Equation 6), question (i) appears straightforward. If there are fitness differences that are associated with parental trait differences, then the trait will change (unless the first term is exactly counter-acted by the second term) and it seems reasonable to call this "change due to natural selection". That is, natural selection of the trait is causing part of the total trait change. The answer to question (ii), on the other hand, is not obvious from examining Equation 6. As is well known, a covariance between a trait and fitness can arise without the trait in question causing the differences in fitness because a trait can change due to natural selection for another (correlated) trait. If the question of interest is (ii), then

Equation 6 does not tell the whole story. Equation 6 only makes clear whether or not selection could be for trait z; additional methods such as contextual analysis (e.g., Lande and Arnold 1983; Heisler and Damuth 1987) are needed to parse out causation from mere correlation. The basic idea of contextual analysis is to construct a regression of fitness on traits. Each trait can then be associated with a partial regression coefficient that captures its effect on fitness when correlations with other traits are controlled for. The regression can also be combined with the Price approach to get a single-level partition corresponding to "selection of" versus "selection for" the trait in question. For multi-level cases, characteristics of the group to which an individual belongs are considered contextual characteristics of that individual. Non-zero partial regression coefficients for contextual characters indicate that the group affects individual fitness. Whether we are considering a single or multi-level case, if we want to say "natural selection for trait z is causing change in population mean z", then the standards of evidence are higher than just saying "population mean z is changing due to natural selection for something". Statements of the former type are needed if our interest is in z as a potential adaptation. If there is natural selection for (rather than of) z, then we would expect (all else equal) that the change in mean z was adaptive.

For more discussion on the relationship between the Price and contextual (and related) approaches see: Okasha 2004, Godfrey-Smith 2008, Sober 2011, Frank 2012, Okasha and Paternotte 2012. Here we simply note that the motivation behind the two approaches can be very different. With the original Price partition, one is interested in contrasting change that stemming from associations between traits and fitness with change due to biased inheritance of traits. With the contextual (and related) approaches, one is interested in distinguishing change in a trait that is

due to its own effect on fitness from change that is due to its correlation with another trait that affects fitness in a population.

Levels of selection and levels of adaptation in volvocine algae

For our volvocine algae-inspired model, we are interested in extending statements about natural selection *for* (rather than *of*) a trait to a hierarchical setting. For example, if we considered a population corresponding to scenario B in which there was variation in average g values of different colonies, there would be positive covariance between group-level fitness and group mean g value. However, this only tells us that in scenario B, there is the formal appearance of group selection *of* group mean g value. We need to grapple with whether there is group selection *for* group mean g value in order to address the issue of group mean g value as a potential group adaptation.

Imagine we had organisms corresponding to each scenario: A, B, C1, and C2. Parameter values are those described in Figure 2. For each organism (A, B, C1, and C2), we seed a test tube with equal proportions of two types of individuals: individuals with genotypes corresponding to $g = 0.8$ and individuals with genotypes corresponding to $g = 0.9$. (We ignore mutations in g.) Suppose we observe, as Figure 2 predicts, that after a time only one type of individual remains in each test tube: $g = 0.8$ individuals for the test tubes corresponding to scenarios A, B, and C1 and $g = 0.9$ individuals for the test tube corresponding to scenario C2. In all cases, the individuals with the better value of g came to dominate the population to the exclusion of the individuals with the more suboptimal value of g. The issue of whether one prefers to do the fitness accounting by considering the births and deaths of cells or by considering the births and deaths of colonies is irrelevant. Recall that $r_L^A \equiv r_L^B \equiv r_H^B$ and $r_L^C \equiv r_H^C$.

This thought experiment highlights a point of tension in extending the concept adaptation to a hierarchical level. Recall that an adaptation can be recognized as a trait value that is predicted from an optimality model (Parker and Maynard Smith 1990). By this idea, $g = 0.8$ in scenario C1 (which is the same value as in the B scenario) is an adaptation, but is it a group adaptation? Using the Price approach to distinguish higher level selection from selection due to frequency changes within groups tends to be associated with the assumption that "individual level selection" simply means "within-group individual selection" in multi-level scenario. That is, when considering multi-level scenarios, individual selection occurs "precisely when there is fitness variation within groups" (Sober and Wilson 2011). That kind of individual level selection is not happening in the scenarios we are considering because all groups are homogenous. This line of thinking would lead one to conclude that scenario C is a case of only group selection and not a multi-level selection scenario (Table 2). This would indicate that $g = 0.8$ in scenario C1 should be considered a group adaptation. It is adaptive because of the optimality analysis; it is a group adaptation because only group selection is occurring. However, this conclusion is potentially problematic. The same trait value evolved in scenario B (the cross-level byproduct case). The results we observed in C1 are the same as our expectation of what the results would be, given that cells were arranged into meaningless groups. More generally, we could make scenario C1 arbitrarily close to scenario B by adjusting α and β to reflect an extremely minor group effect. Does it make sense to call $g = 0.8$ in population C1 a "group adaptation" when $g = 0.8$ in population B must be more properly called a "cell adaptation"? (Table 3)

We will distinguish the evolution of $g = 0.8$ in the scenario C1 population from $g = 0.9$ in the scenario C2 population by calling the latter a "group-specific adaptation" and calling the former a "byproduct group adaptation". Group-specific adaptations not only result from

maximization of group level fitness (r_H^C), but also must be substantially different from trait values that would maximize colony fitness (r_H^B) in the absence of colony-dependent trait-fitness relationships. Equivalently (in this model), they are substantially different from trait values that would maximize lower-level fitness if the groups were dissolved (r_L^A). (Recall that $r_L^A \equiv r_L^B \equiv r_H^B$.) We will further discuss the implications of this point in the next section.

We define counterfactual relative fitness, ω, to refer to the relative fitness that a scenario-C-type group would have had (given particular trait values) if there were no group-dependent effects (i.e. $\omega_i \equiv \frac{(r_H^B)_i}{\mathrm{E}(r_H^B)}$, where $\mathrm{E}(r_H^B)$ denotes the mean fitness in a population and i indexes the members of the population). For the model developed here, counterfactual fitness is the fitness value embodied in both scenarios A and B (because $r_L^A \equiv r_L^B \equiv r_H^B$). Note that the "unicellular" trait-fitness relationships that dominate in population C1 are not absent from population C2 (see Figure 3). Rather, additional group-dependent trait-fitness relationships make a difference to the outcome in population C2 (the evolution of $g = 0.9$). Both kinds of relationships (the group-dependent and the group-independent effects of g) contribute to the fitness of the colony in scenario C.

With an interest in group-specific adaptation in mind, we return to the issue of partitioning the total mean trait change ($\Delta\bar{z}$) into biologically meaningful components. We now focus on the C1 and C2 populations described above and consider the trait value (z) of each colony to be its mean g value. (Groups are homogenous, so this is also just the g value of each cell.) The relative fitness of a colony is given by $w_i = \frac{(r_H^C)_i}{\mathrm{E}(r_H^C)}$ (where r_H^C is equal to r_L^C, which is defined in Equation 5 and $\mathrm{E}(r_H^C)$ is the average value of r_H^C for a population of colonies whose members are indexed by i). We assume that a colony's fitness is determined only by its g value

and that the transmission of g value between the generations is perfect ($\Delta g_i = 0$ for all i). Thus, we can account for change in mean g with the first term of the Price equation:

$$\Delta \bar{g} = \mathrm{Cov}(w_i, g_i) \tag{7}$$

In order to isolate change leading to group adaptation, we are concerned with correcting for the group-independent effects of g, that is, correcting for the effects that do not depend on group membership. The group-independent effects of g are captured in counterfactual relative fitness, ω. Counterfactual relative fitness is the group relative fitness that would be attained if g had only group-independent effects (Equation 3). Recall that in the populations under consideration, g does have effects that depend on there being a group level of organization. Actual absolute fitness (r_H^C, Equation 5) and counterfactual absolute fitness (Equation 3) are different functions of the trait under consideration (g). For these scenarios, counterfactual fitness of a C-type colony is the fitness that the colony would have attained with the same g value if scenario B assumptions had held. We define $\Delta w_i = w_i - \omega_i$ as the difference between the actual fitness and counterfactual fitness and incorporate counterfactual fitness into Equation (7) as follows:

$$\Delta \bar{g} = \big(\mathrm{Cov}(w_i, g_i) - \mathrm{Cov}(\omega_i, g_i)\big) + \mathrm{Cov}(\omega_i, g_i) \tag{8}$$

$$\Delta \bar{g} = \mathrm{Cov}(\Delta w_i, g_i) + \mathrm{Cov}(\omega_i, g_i) \tag{9}$$

The second term in Equation 9 corresponds to the trait change that would have happened if the groups were biologically meaningless collections of unicellular individuals. The first term is the additional trait change that is directly attributable to the group-dependent trait-fitness relationships (Table 4). This partition can be very different for the C1 vs. C2 population (depending on the values of g that are present in the initial population; Table 5). This partition captures the difference between a byproduct group adaptation (what we expect to evolve in C1

when test tubes were seeded with $g = 0.8$ and $g = 0.9$ individuals) and a group-specific adaptation (what we expect to evolve in C2 when test tubes were seeded with $g = 0.8$ and $g = 0.9$ individuals). We tentatively call the change represented in the first part of Equation 9 "change due to group-level selection for g" and the change represented in the second part of Equation 9 "change due to global individual-level selection for g" (Table 4). Note that both terms reflect "selection for" not "selection of" g. "Global individual selection" should not be confused with within-group selection, which by assumption is not occurring in our scenarios.

Presumably, it would be possible to analyze situations in which all three categories (group specific selection, global individual selection, and within-group selection) were non-zero. However, some conceptual subtleties become apparent when thinking about scenarios with non-zero within-group selection. Mechanistically, there seems to be little difference between the effect that a sub-optimal (from lower-level optimality analysis) trait value would have in a mixed-phenotype colony compared to the effect that it would have when homogenous scenario-B-type colonies compete with one another. That is, both "global individual selection" and "within group selection" refer to the same thing: cell-cell competition that is resolved on the basis of group-independent cell trait(s). This suggests that separating their effects on trait change may not be desirable or possible. Only the context of the competitive interactions distinguishes global individual from within-group selection. Whereas "global individual selection" refers to the global competition among cells that just happen to be arranged into spatio-temporally discrete groups, "within-group selection" refers to local competition among cells within a spatio-temporal group. Here we suggest that global individual and group specific selection are separable causes of trait change when within-group selection is absent. However, the separability of global individual, within-group, and group specific selection when all three are at play is not yet clear.

The advantage of calling only the first term in Equation 9 "change due to group-level selection for g" is that it hierarchically extends the strong link between selection and design. By this conception, group-level selection *for* a trait produces *specifically* group-level design in that trait. Natural selection is the only process known to produce the appearance of design; group-level natural selection is the only process known to produce the appearance specifically of group-level design. Only group selection for g—that is a group-level cause effect relationship between the trait and fitness—can cause the evolution of design that is specific to the group level. This point echoes Heisler and Damuth's (1987) emphasis on the hierarchical level of the trait-fitness relationship. The advantage of calling the second term "change due to global individual-level selection for g" is that it maintains a continuity with the intuitive interpretation of a scenario B-type of case. We do not want to say that "group selection" is occurring in scenario B. Only "global individual selection" is occurring in scenario B. Scenario C1 is only slightly different from scenario B. Therefore, it is appealing to describe trait changes in scenario C1 as being mostly, though not entirely, driven by "global individual selection". This point was also made by S. Okasha as described in Sober (2011).

The partition in Equation 9 concerns how much trait change is adaptive at the specifically group level versus at the global cell level. Alternatively, it is clear that consideration of equilibrium values in this simple example could give a quantification of the extent to which an evolved trait value is an adaptation. That is, here we consider the equilibrium end-point (the adaptation) rather than the process leading there (the adaptive trait change). The equilibrium value (i.e. the value that maximizes fitness) for g is 0.274 in scenario B, 0.281 in scenario C1, and 1 in scenario C2. The C1 value exceeds our cross-level by-product (scenario B) expectation by about 2.5% whereas the C2 value exceeds our cross-level by-product expectation by about

265%. This simple example shows how an explicit model that incorporates unicellular trait-fitness relationships and specific assumptions about the colonial life cycle (i.e., the scenario B model) allows for quantitative support of intuitions concerning the level of adaptation. If we were to observe $g = 1$ (in a hypothetical C2 population that has gone to equilibrium), this trait is *more* of a group-specific adaptation than $g = 0.281$ in a similar C1 population. The extent to which g is a group specific adaptation is a matter of degree and is quantifiable.

It may be objected that attaching "group selection" to the production of *specifically* group-level design is unnecessarily restrictive. That is, in both populations C1 and C2, the value of g changed in a way that can be explained by the relationship between g-value and the reproductive success of colonies. Thus, there is an appeal to the idea that both C1 and C2 are simply cases of only group selection and any adaptive evolution that results are simply group adaptations. Sober and Wilson (2011) seems to take this view by using a trait group definition of groups (to avoid the problem with scenario B) and using a standard Price partition to qualitatively define the meaning of group selection. The problem with this view is that if the definition is interpreted quantitatively, it leads to (at least somewhat) counter-intuitive conclusions in a case of very minor group effect (e.g., C1). By this line of thinking, trait change that mostly would have happened even without the groups should be considered to be due to group selection (and thus contributes to group adaptation). Sober (2011) mentions this problem.

Gardner and Grafen (2009) also endorse defining group selection using the traditional Price partition. They also take the view that when transmission bias is absent (i.e., when all trait change is captured by group-level trait-fitness covariance), the resulting trait change is adaptive at the group level. Clonal groups are one way that transmission bias (within-group selection) can be absent. Our view, however, is that even for clonal groups in which all trait change stems from

between-group trait-fitness covariance, it is possible that the adaptive value of the trait change is not specific to the group level.

For some types of questions, the approach described in Sober and Wilson (2011) may be sufficient; that is, the hierarchical level of the trait-fitness relationships, which causes the distinction between "group specific adaptations" and "group byproduct adaptations" is not necessarily always an important distinction. However, we argue below that for some purposes, especially when considering ETIs, the distinction between "group specific adaptation" and "group byproduct adaptation" is important.

Group-specific adaptations can lead to the evolution of group cohesion

The difference between group specific adaptations and group byproduct adaptations is relevant to the issue of ETIs, that is, to evolutionary transitions from a group with quasi-independent parts to a tightly integrated group with interdependent parts (i.e., a higher-level individual). As group-specific adaptations evolve, the fitness of a group, given some trait value, diverges from what it would have been with the same trait value if the groups were purely spatio-temporal aggregations with no fitness consequences. This also means that the fitness consequences of a trait that is a group-specific adaptation depend on there being a group structure rather than a global population. Consider the trait of producing a mixture of germ- and soma-specialized cells upon cell divisions versus producing only undifferentiated cells. The fitness of a germ-soma differentiated *Volvox* colony may be quite high (or, equivalently, the inclusive fitness of a cell within a *Volvox* colony may be quite high). However, if one imagines dissolving the colony structure, so that there is a global population of cells, a genotype that spawns both germ- and soma-specialized cells would be expected to do very poorly in competition with a genotype that gives rise to undifferentiated cells. Thus, the trait of producing specialized (germ and somatic)

cells is almost certainly a group-specific adaptation and a germ-soma differentiated colony is an extreme example of the interdependence of cells within a colony. Now consider a scenario-C2-type cell (i.e. a cell in a population with groups that really make a difference to cell fitness). The evolution of $g = 1$ genotypes from ancestors that had $g = 0.27$ genotypes would constitute an increase in cell interdependence, even though there is no functional specialization of cells within a colony. Compared to cells with $g = 0.27$ genotypes, cells with $g = 1$ genotypes that were forced to live alone would have lower fitness. That is, the $g = 1$ cells depend on group structure (i.e., on one another) for their fitness. In the germ-soma differentiated colony, the interdependence of cells is more extreme. Without the group context, both germ and somatic cells would be expected to do very poorly. However, even in the absence of cell specialization, something as simple as evolving a $g = 1$ genotype rather than a $g = 0.27$ genotype decreases that genotype's competitiveness in a purely cell-level population and increases the dependence of the $g = 1$ genotype on the group context. The process of cells loosing autonomy (higher-level individuation) could be borne out gradually in undifferentiated colonial volvocine algae, with the evolution of cells specialized in major fitness components (germ and soma) being the end of the spectrum. The beginning of the spectrum need not have anything to do with specialization of fitness components. The evolution of group-specific adaptations could create selective pressure for decreased cell autonomy in completely undifferentiated colonies. The way in which traits related to colony cohesiveness and other fitness-affecting traits (e.g. allocation to growth) co-evolve is not addressed directly by this simple optimality model.

Whether or not the term "group specific adaptation" is a good way to describe something like the value of $g = 1$ in population C2, the distinction between the predicted outcome of selection in C1 versus C2 is important for the following reason. Imagine that both populations

C1 and C2 were transferred to an environment that dissolves the connections which previously held colonies together. The cells that evolved in C1 would be well adapted and the environmental change would not constitute a substantial new selective pressure. By contrast, independent cells derived from population C2 are no longer well-designed for life as independent cells. Therefore, an environment which tends to break up colonies constitutes selective pressure for stronger mechanisms of holding colonies together, i.e. for higher group cohesion, in population C2. Thus, the adjustment of g (or any trait) to correspond to specifically group-level constraints—the feature characteristic of C2 and not C1—is likely an important factor in the evolution of group cohesiveness. Cohesiveness at the group level is an important aspect of an evolutionary transition. It constitutes a change in the population structure towards the new higher level unit and thus could have effects on the evolution of many other traits.

Evolution of a group life cycle

The analysis above of group-level adaptation in simple colonial volvocine algae helps us understand the origin of group life cycles and provides one resolution to the chicken-and-egg problem of group reproduction. The problem is that group selection typically presupposes an existing group-level life cycle; yet, many features of group life cycles appear to be adaptations, i.e., products rather than pre-requisites of group-level selection (Griesemer 2000). We think that the establishment of a life cycle at the group level could proceed according to the following steps:

1. Features (such as palintomy) that facilitate the emergence of group structure are already present in unicells.
2. Group structure emerges.

3. Group-specific adaptations evolve in traits (such as g) that were already subject to genetic regulatory mechanisms in the unicellular context.
4. These group-specific adaptations lead to selective pressure for increased cohesiveness or regularity of the group structure.
5. Further evolution of group-specific life cycle adaptations as group selection on cohesive groups is now established.

These steps are formulated with the volvocine algae in mind and we do not think that they all generalize to other systems. In the volvocine algae, palintomy allows for a very small change in the adhesiveness of daughter cells to produce a rudimentary group-level life cycle (steps #1 → #2; Fig. 1). Group-specific adaptations, for example, different ways of regulating growth (g) and the number of rounds of cell divisions per palintomic cycle (n), could evolve in response to this initial, rudimentary group life cycle (step #3). Lower-level traits that have novel effects in a group context and were subject to regulation prior to the emergence of group structure (possibly like g or n) are likely to already have genetic regulatory mechanisms which can be polymorphic in the ancestral population or can be tweaked by mutation in regulatory elements. Thus, these kinds of traits should respond quickly to group-level selection. As soon as the group structure emerges, such traits affect group fitness and have group-level heritability. They were presumably already heritable at the cell-level and for a group life cycle involving small clonal groups with a unicellular bottleneck, heritable differences among cells automatically are translated into heritable differences among colonies. Group-specific adaptations in traits like g or n create selective pressure for further group cohesiveness, a change which can be seen as solidifying the group-level life cycle (step #4).

Once the group life cycle is better established (due to increased cohesion of the groups), the stage is set for an eventual response to group selection of traits that did not show regulated variation in the unicellular ancestor (i.e. traits that cannot arise from mutations in "easy" targets). In the volvocine algae, an example of a trait change that shows up only in the later-branching colonials (rather than all colonials) is the rotation of the flagella according to position in the colony (Herron and Michod 2008). Unicellular ancestral populations were presumably fixed for the non-ability to rotate flagella; no regulatory mechanisms were necessary to achieve this phenotype. Therefore, this kind of trait is not a good candidate for step #3, but can be explained by group selection after the feedback between step #3 and step #4 established a reliable group life cycle. Depending on the details of the biological example, it is possible that steps #1–5 could occur while group sizes remained very low and before the evolution of germ-soma specialization. This would mean that the issue of conflicts between within-group selection and between-group selection would crop up later in the transition from unicellularity to multicellularity rather than at the outset. Rather than immediately being threatened by the effects of selection from below, group adaptations have time to become established in the absence of such conflict. Only when group size becomes larger do the potentially highly integrated, well-adapted groups face the problem of potentially disruptive effects of within-group cell level selection. This contrasts sharply with the immediate conflict between the two levels that emerges when different assumptions are made about the origin of the group life cycle (e.g. when group reproduction originates from fragmentation (Roze and Michod 2001)).

Another interesting upshot of steps 1–5 is that the group adaptations that become established during this phase could be needed for the advantages of larger size to be realized. That is, growing larger may not be a viable option until mechanisms of integration and cohesion

have evolved in smaller colonies. This makes intuitive sense, as high degrees of integration among cells is often necessary to reap the benefits of larger size, whereas many of the costs of larger size which must be overcome for size to increase (local depletion of resources and accumulation of waste products, etc.) do not depend on integration among cells.

One candidate for an early trait change that could have been adaptive specifically at the group (colony) level in volvocine algae is a change in the regulation of n, the number of rounds of cell division per palintomic cycle (Kirk 2005; Herron and Michod 2008). The species-specific n for unicellular *C. reinhardtii* has been suggested to range from 1–5 whereas the maximal n for some colonial species appears to be less than 5 (Kirk 2005). However, differences in n-value among volvocine species have yet to be documented rigorously across a range of realistic environments. Furthermore, the exact relationships of n to the sizers, timers, and environmental cues that underpin it are still controversial. For example, the number of rounds of division per cycle is set by the ending mother cell size, but what sets the ending mother cell size? Some groups describe an effect of light intensity, with faster growing cells (higher light intensity) generally ending growth sooner (Vítová et al. 2011; Matsumura 2010). Other groups emphasize that the environmental switch from light to dark can cause the initiation of divisions, making final mother cell size a side-effect of when the light period ends (e.g. Voigt and Münzer 1987 and Oldenhof et al. 2006). Both views are compatible if cells divide in response to reaching a maximal size if it is reached and otherwise divide in response to a light-dark transition. Clarity concerning the regulatory underpinning of unicellular *C. reinhardtii* n value is important for understanding regulation of n as a possible group adaptation. Strong evidence for n-value regulation as a group adaptation in early-branching colonials would come if one had colonial strains that differed in n-value regulation. The strain with the more unicellular-like n-value

regulation should win in competition if the colony structure is dissolved or disrupted whereas it should lose in competition if the colony structure is intact. If regulation of n is a primarily group-level adaptation in the earliest-branching colonials (*Basichlamys* and *Tetrabaena*), this raises the question of why these colonies nevertheless have such low cohesiveness. There is much more to explore with respect to the connections between group adaptations and the evolution of group cohesiveness.

Conclusions

Extending the fundamental concepts in evolutionary biology—including natural selection, fitness, and adaptation—to apply unambiguously in cases with hierarchical structure is a major and ongoing challenge. It is of great importance because we need these concepts to understand not only how evolution occurs in hierarchically structured populations, but also to understand how and why such hierarchical structure has repeatedly emerged.

By considering the volvocine algae, we have encountered some challenges as well as resolved some ambiguities in applying the concepts of natural selection and adaptation to the group level. It is not clear whether clonal groups that are only mildly influenced by the effects of the group structure on fitness components should be considered to be evolving entirely by group-level natural selection (Table 2). Similarly, it is not clear whether traits that are optimal for such colonies should be considered "group adaptations" (Table 3).

It is clear, however, that distinguishing cases of mild group effect from cases of strong group effect is important for understanding the implications of different levels of group cohesiveness. Cases of strong group-effects are likely to lead to group-specific adaptations (i.e., trait values that are adaptive because of the group context). When group-specific adaptations evolve, they create selective pressure for enhanced group cohesiveness and group-level

individuality. The loss of autonomy of cells as they gain group-specific adaptations can occur even in the absence of cellular differentiation.

We began to develop one approach to quantifying the distinction between trait changes that contribute to group specific versus group byproduct adaptations. The approach hinges on consideration of what we call counterfactual fitness, the fitness that groups would have if the trait in question had no group-dependent fitness effects (Table 4). By modeling the fitness of a colony as a function of both a trait value and counterfactual fitness (given that same trait value), we can separate the group-dependent and group-independent effects of a trait. However, more development would be needed to justify or invalidate the assumptions about separability of causes that are necessary for this approach. Combining an explicit unicellular-based model with the Price equation allows for quantification of the trait change stemming from group independent effects (and presumably leading to group byproduct adaptations) versus the trait change stemming from group dependent effects (and presumably leading to group specific adaptations).

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Tables

Table 1. Description of symbols used.

	Description	Dimension	Constraints
$A; B; C$	Used as a superscript to refer to a scenario.		
$c;\ c_i; c_{min}$	Initial size of a cell (fixed); initial cell size where i indexes generation; size at which the cell is too small to undergo a cell division.	Mass	> 0
F	Fecundity. Number of offspring per individual per generation, conditioned on survival.		≥ 0
g	Proportion of total cell energy or resources invested in growth.		$0 < g < 1$
$H; L$	Used as a subscript to refer to the higher and lower (respectively) hierarchical level of organization.		
k_a	The actual cell growth rate of a cell with a particular g-value.	Mass Time^{-1}	≥ 0
k_m	The cell instantaneous mortality rate that would occur if all energy or resources were devoted to growth ($g = 1$).	Time^{-1}	> 0
k_g	The cell growth rate that would occur if all energy or resources were devoted to growth ($g = 1$).	Mass Time^{-1}	> 0
M	Instantaneous mortality rate (of a cell or colony).	Time^{-1}	≥ 0
n	Number of rounds of cell divisions per palintomic cycle.		positive integer
q_i	Frequency of parental type i in a population.		
r	Intrinsic rate of (cell or colony) population increase.	Time^{-1}	
t_s	Amount of time each generation during which growth occurs.	Time	$0 < t_s < T_t$
T_t	Generation time. Average time between birth of an individual (cell or colony) and the birth of the offspring of that individual. Because we are only concerned with semelparous organisms here, this is also the average lifespan of a cell/colony.	Time	$T_t > 0$
w_i	Relative fitness of parental type i in the Price equation approach.		≥ 0
W	Absolute fitness.		≥ 0
x	Probability that a cell survives one generation.		$0 < x < 1$
y	Value of a trait that is correlated with a trait of interest, z.		
z	Value of a trait of interest in the Price equation approach.		
α	Used in specifying the effect of colony cell number on cell mortality rate.		> 0

β	Used in specifying the effect of colony cell number on cell mortality rate.		> 0
ω	Counterfactual relative fitness. The relative fitness that a scenario-C-type group would have had if there were no group-dependent effects of the trait.	Time^{-1}	≥ 0

Table 2. Levels of selection in the volvocine algae models.

	Global individual selection?	Within-group individual level selection?	Group selection?
A	Yes	No	No
B	Yes	No	No
C1	?	No	Yes
C2	?	No	Yes

Table 3. Hypothetical evolution of g and its relationship to optimality and adaptation. Here we are assuming that test tubes corresponding to each scenario (A, B, C1, and C2) were seeded with equal proportions of two genotypes: a $g = 0.8$ genotype and a $g = 0.9$ genotype. After some time, one genotype came to dominate in each test tube; this is the "hypothetical value of g". Parameter values are those given in Figure 2.

Population	Hypothetical value of g	Value of g is a group adaptation?
A	0.8	No
B	0.8	No
C1	0.8	?
C2	0.9	Yes

Table 4. Partitioning of trait change into components that could be attributable to different categories. The traditional Price equation partition is given in the first row. The second row corresponds to a combination of contextual analysis and the Price equation. The purpose of this partition is to distinguish "selection of" from "selection for" in a single-level case. Absolute fitness (W) is modeled as a function of two traits (z and y). β_{Wz} is the partial regression coefficient of z on W and β_{Wy} is the partial regression coefficient of y on W. See Frank (2012) for a full description of this partition. Here, we assume that the trait of interest has cause-effect relationships with fitness, making the "selection of z" term equal to zero in the row corresponding to our proposed partition. The proposed partition (third row) distinguishes the effects of group-dependent trait-fitness relationships from the effects of group-independent trait-fitness relationships. Both kinds of relationships constitute "selection for", though the hierarchical level of these two kinds of "selection for" is contested.

Price partition		Change due to shifts in numerical dominance of a type's decedents:			Change due to bias in transmission of types:
	$\Delta\bar{z} =$	$\mathrm{Cov}(w_i, z_i)$		+	$\mathrm{E}(w_i \Delta z_i)$
		Change due to cause-effect relationship between z and W:		Change due to correlation between z and y plus cause-effect relationship between y and W:	
Single-level contextual analysis plus Price partition	$\Delta\bar{z} =$	$\frac{\beta_{Wz}\mathrm{Var}(z)}{\bar{W}}$	+	$\frac{\beta_{Wy}\mathrm{Cov}(y,z)}{\bar{W}}$	0
		Change due to group-dependent cause-effect relationship between z and w:	Change due to group-independent cause-effect relationship between z and w:		
Proposed partition	$\Delta\bar{z} =$	$\mathrm{Cov}(\Delta w_i, z_i)$	+ $\mathrm{Cov}(\omega_i, z_i)$	0	0
Tentative label for partition:		Specifically group-level selection for z	Global individual-level selection for z	Selection of z	Within-group selection of/for z

Table 5. Partitioning of trait change under two hypothetical kinds of groups ($\beta = 50$ and $\beta = 1$) and two hypothetical starting populations. In the first row, a starting population of equal proportions of $g = 0.1$ genotype cells and $g = 0.2$ genotype cells is considered. The amount of trait change (Δg, Equation 7) is calculated by assuming perfect transmission of traits. The group specific change (the first term in Equation 9) is divided by the global individual change (second term in Equation 9). The absolute value of this gives the relative magnitude of the change caused by the group specifically compared to the magnitude of change that would have happened even without the group. Equation 3 is used to calculate w and Equation 5 is used to calculate ω. Parameter values are those given in the Figure 2 caption. For each starting population, a case of minor overall group effect ($\beta = 50$) is compared to a case of major overall group effect ($\beta = 1$). When the starting g values are small, the difference between the two cases is not great. (Also see Figure 2.) Both specifically group selection and global individual selection favor the $g = 0.2$ genotype and both processes contribute to an increase in the trait value (positive Δg). When the starting genotypes are large, global individual level selection favors the $g = 0.8$ genotype (negative sign of global individual level term) whereas specifically group selection favors the $g = 0.9$ genotype (positive sign of specifically group level term). When the group-level effect is small ($\beta = 50$), the ratio of the terms is less than 1, indicating that global individual level selection dominates and the net trait change is negative. Conversely, when the group-level effect is large ($\beta = 1$), the ratio of the terms is greater than 1, indicating that specifically group level selection dominates and the net trait change is positive.

	Minor overall group effect ($\beta = 50$)				Major overall group effect ($\beta = 1$)			
	Δg	$\left\lvert\frac{\text{group specific change}}{\text{global indiv. change}}\right\rvert$	Sign of group level specific term	Sign of global individual level term	Δg	$\left\lvert\frac{\text{group specific change}}{\text{global indiv. change}}\right\rvert$	Sign of group level specific term	Sign of global individual level term
Population with low g values ($g = 0.1$ and $g = 0.2$)	3.3×10^{-4}	0.016	positive	positive	5.0×10^{-4}	0.520	positive	positive
Population with high g values ($g = 0.8$ and $g = 0.9$)	-2.2×10^{-4}	0.114	positive	negative	8.1×10^{-5}	1.320	positive	negative

Figure Legends

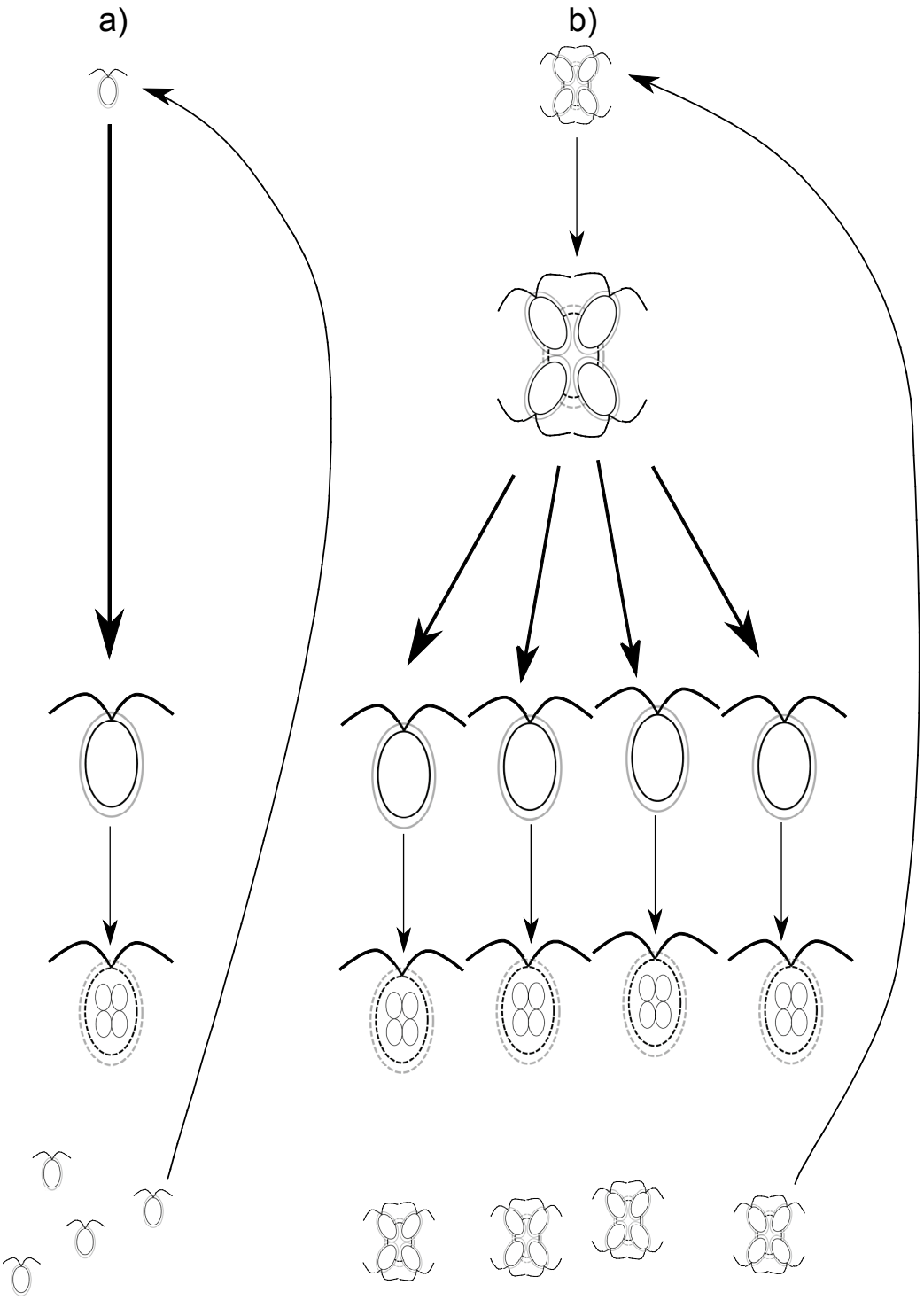
Fig. 1 a) Life cycle of *Chlamydomonas reinhardtii*. b) Life cycle of *B. sacculiferum*. Here $n = 2$. Cells remain attached for a time as they grow, then separate prior to cell division. After Iyengar and Desikachary (1981). All cells within a colony are assumed to be genetically identical. A mutation that leads offspring cells to temporarily adhere to the mother cell wall material can cause a transition from the unicellular life cycle (left) to the colonial life cycle (right)

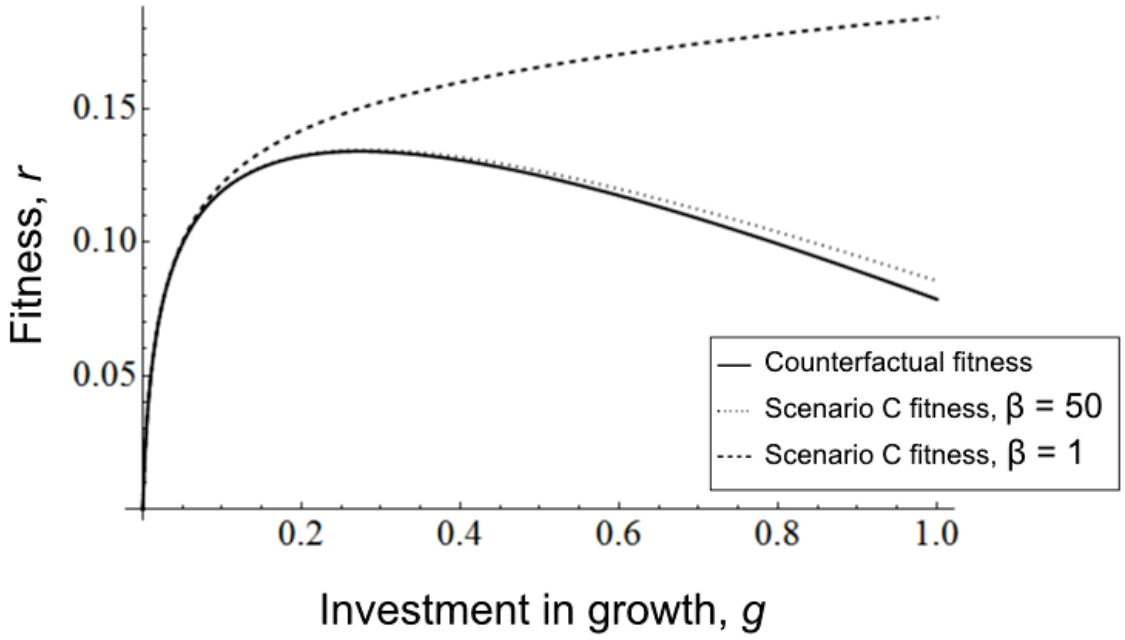
Fig. 2 Fitness as a function of cell investment in growth (g). The counterfactual fitness (solid line) is the fitness a colony would have (given a g value) if there were no group-dependent effects of g (calculated from Eqn. 3 and A6). The parameter values are: $T_t = 24$, $t_s = 12$, $k_m = 0.15$, $k_g = 20$, $c_{min} = 1$. The dotted line and dashed line show fitness as a function of g for Scenario C (Eqn. 9). For the dotted line, $\alpha = 0.01$ and $\beta = 50$ (minor group effect); for the dashed line, $\alpha = 0.01$ and $\beta = 1$ (major group effect)

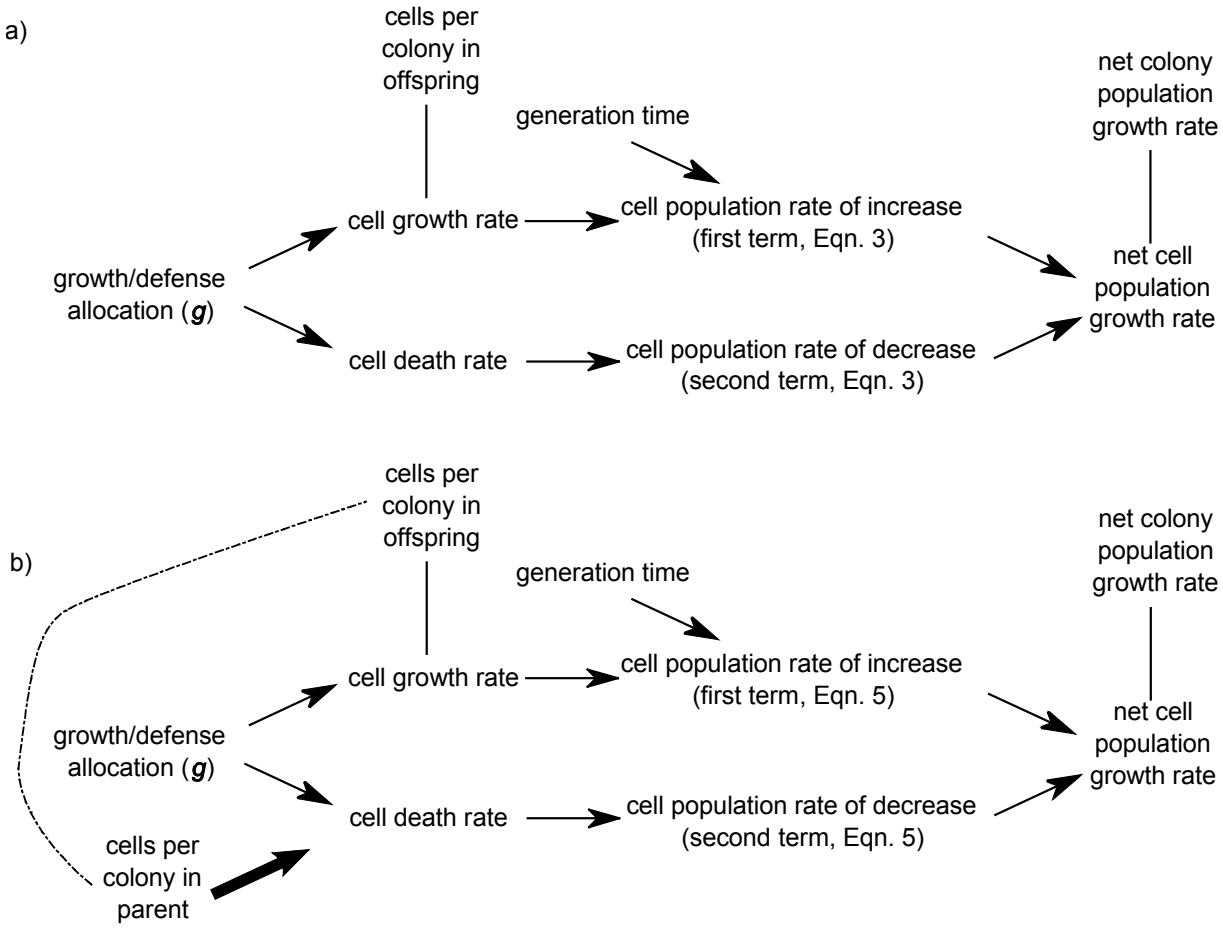
Fig. 3 a) Causal relationships modeled in scenario B, the cross-level byproduct scenario. Both the cell growth rate (rate of mass increase) and the cell death rate are determined by a cell's allocation of limited resources or energy (g). The cell growth rate ($k_g g$), together with other aspects of the unicellular life cycle which are assumed to be fixed (c, T_t, t_s), determines the rate at which the population of cells grows (first term, Eqn. 3). The cell death rate ($k_m g$) determines the rate at which the population of cells declines (second term, Eqn. 3). These cause-effect relationships are shown as arrows. In Scenario B, the investigator can recognize spatio-temporally distinct groups of cells (colonies). Therefore, colony-level properties can be recognized; these are shown by the vertical lines. To see how cell growth rate (along with other factors) gives rise to offspring cells per colony, refer to Eqn. A4. Eqns. A10-A21 show how the net colony population growth rate arises from the cell-level cause-effect relationships. b) Causal

relationships in scenario C, the genuine group selection scenario. Note that the only difference between scenario B and C is that in scenario C, the number of cells per colony affects the mortality rate of the cells. This cause-effect relationship is shown by a bold arrow. In scenario C, the cell growth rate ($k_g g$) has both a direct, group-independent effect on cell population growth rate (first term in Eqn. 5) as well as an indirect, group-dependent effect on cell population rate of decline (second term in Eqn. 5). The broken line indicates that the number of cells per colony in a focal colony arises in part from its parent's cell growth rate.

Figures




Fitness, r
0.15
0.10
0.05
0.2
0.4
0.6
0.8
1.0
Investment in growth, g
Counterfactual fitness
Scenario C fitness, β = 50
Scenario C fitness, β = 1


a)
cells per colony in offspring
generation time
net colony population growth rate
cell growth rate
cell population rate of increase (first term, Eqn. 3)
growth/defense allocation (g)
net cell population growth rate
cell death rate
cell population rate of decrease (second term, Eqn. 3)
b)
cells per colony in offspring
generation time
net colony population growth rate
cell growth rate
cell population rate of increase (first term, Eqn. 5)
growth/defense allocation (g)
net cell population growth rate
cell death rate
cell population rate of decrease (second term, Eqn. 5)
cells per colony in parent

Appendix 1

Cell-level fitness, scenario A

We assume that the generation time (T_t, the total time between rounds of divisions) is simply 24 hr. This is consistent with behavior of *C. reinhardtii* cells in some culture conditions. The rate of mortality of cells (M_L^A) is assumed to depend on the available resources as well as at the amount of cell's investment of limited resources in machinery for growth:

$$M_L^A = k_m g \tag{A1}$$

Here k_m is the mortality rate that would occur if the cell invested all limiting resources in growth (as opposed to defense), and g is the proportion of limiting resources the cell invests in growth rather than defense. We assume that the actual growth rate of a cell is given by:

$$k_a = k_g g \tag{A2}$$

where k_g is the ideal cell growth rate (i.e., the cell growth rate that would occur in the given conditions if the cell invested completely in the growth machinery). The amount of growth that happens in one generation is offset by the number of divisions such that:

$$c_0 + k_a t_s = 2^{n_0} c_1 \tag{A3}$$

Here, c_0 and c_1 are the initial cell sizes of the parent and offspring, respectively, k_a is the actual rate of cell growth (as defined above), t_s is the amount of time that a cell was able to grow (roughly corresponding to the sunlight hours), and n_0 is the number of rounds of divisions per cycle that the parent undergoes. Because n must be an integer, the initial offspring cell size is not necessarily the same as the parent initial cell size. In *C. reinhardtii*, the rounds of cell divisions continue the maximum number of times so as to result in offspring that are larger than some minimum threshold size, c_{min}. The smallest possible offspring size is c_{min} and the largest possible offspring size is just under $2c_{min}$ (because cells that are at least $2c_{min}$ undergo another

round of divisions). The number of rounds of divisions, n, is the largest previous integer (the floor) of the number of divisions that would have resulted in exactly c_{min} for an offspring size:

$$n_0 = \text{Floor}\left[\log_2\left[\frac{c_0 + k_g g t_s}{c_{min}}\right]\right] \quad \text{(A4)}$$

In this model, the cell is converting continuous improvement in growing time or growth rate into discrete improvements in reproduction (n) by allowing for variation in the starting size of a cell. From re-arranging Eqn. A3, the initial size of a parental cell in a particular generation (with i indexing the generation under consideration) is:

$$c_i = \frac{c_{i-1} + k_g g t_s}{2^{n_{i-1}}} \quad \text{(A5)}$$

That is, the starting size of a cell is determined in part by the starting size of its parent as well as the number of times its parent divided. Similarly, the generation-specific value for n is:

$$n_i = \text{Floor}\left[\log_2\left[\frac{c_i + k_g g t_s}{c_{min}}\right]\right] \quad \text{(A6)}$$

That is, the number of times a cell divides depends on that cell's starting size (which in turn depends in part on the number of times its parent divided, as shown in equation A5). When these recursive equations are considered (not shown), we can see that for fixed environmental conditions, the inter-generational variation in n depends on g, the cell's investment in growth. If growth investment is such that the total growth tends to be near a threshold for a lower or higher n, then we see cycles of varying n values. The genotype can cycle between a lower and higher value of n on the scale of a few generations under fixed environmental conditions. Alternatively, if growth investment is such that the total growth tends not to be near a threshold for a lower or higher n, then the initial cell size (c) and n appear to be stable (at least on the scale of ~50 of generations). In this parameter range, a genotype that invests slightly more in growth only has the effect of making equilibrium starting cell size higher. In this model, differences in starting

cell size are assumed to not affect fitness, so this effect could create a jagged relationship between g and fitness (r).

In order to avoid a jagged relationship between g and fitness, we treated c as a constant and allowed n to vary continuously. The initial size of *C. reinhardtii* cell is known to be very similar for daughter cells originating from mother cells with different final sizes (Donnan and John 1983), so treating c as a constant is reasonable. While non-integer values of n are certainly unrealistic, the smoother fitness curve that results from this is probably more realistic. The actual relationship between growth investment and fitness in *C. reinhardtii* is not critical for the points developed here. Certainly, there are many more details concerning the way in which cell cycle progression is regulated (with downstream effects on n) that are relevant for understanding the status of n-value as a potential group adaptation in volvocine algae. We do not consider these volvocine-specific issues here, instead developing a simple model for the purpose of illustrating and supplementing the discussion presented in the main text.

We model the fecundity of a cell as:

$$F_L^A = 2^n \tag{A7}$$

Substituting from A4 without the "Floor" function and with a constant starting cell size gives:

$$F_L^A = \frac{c + k_g g t_s}{c} \tag{A8}$$

Combining the fitness components, we get:

$$r_L^A = \frac{\ln\left(\frac{c + k_g g t_s}{c}\right)}{T_t} - k_m g \tag{A9}$$

Colony-level fitness, scenario B

The colony-level generation time in scenario B is the same as the cell-level generation time (24 hr.). We have assumed a life cycle in which the generations of the higher and lower level are synchronized (Figure 1). Whereas if a cell survives, it produces 2^n offspring cells, the same is not true of colonies. Because in scenario B we assume that the colonies do not have effects that are relevant to survival and reproduction, cells within a colony survive or die independently of one another. The number of cells alive at the time of reproduction determines the number of offspring colonies a colony will have. We define x to be the probability that a cell survives one generation:

$$x^B = \exp(-k_m g T_t) \tag{A10}$$

Equation A10 follows from the assumption of constant cell mortality rate ($k_m g$). Colonies that have only one surviving cell have only one offspring colony, and so on up to colonies with the maximum number of surviving cells (2^n, where n is defined as before). The overall expected number of offspring colonies for a parent colony is:

$$\sum_{i=1}^{2^n} \binom{2^n}{i} (x^B)^i (1 - x^B)^{(2^n - i)} i \tag{A11}$$

Formula A11 is the mean of a binomial distribution, which is also given by:

$$2^n x^B \tag{A12}$$

The colony-level fecundity is the expected number of offspring colonies, given colony survival (i.e., given that not all the cells in a colony died). Thus, the colony-level fecundity is:

$$F_H^B = \frac{2^n x^B}{1 - (1 - x^B)^{2^n}} \tag{A13}$$

We assume that the rate of colony mortality is constant, so the probability that a colony survives for one generation is $\exp(-M_H^B T_t)$. The probability that a colony survives for one generation is also simply the probability that not all of the cells within that colony die in one generation, or $1-(1-x^B)^{2^n}$, where x^B is defined as in Equation A10. This gives:

$$1-(1-x^B)^{2^n} = \exp(-M_H^B T_t) \tag{A14}$$

Rearranging:

$$M_H^B = \frac{-\ln\left(1-(1-x^B)^{2^n}\right)}{T_t} \tag{A15}$$

Combining the fitness components described above according to Equation 2 gives the colony-level fitness (intrinsic rate of population increase):

$$r_H^B = \frac{\ln\left(\frac{2^n x^B}{1-(1-x^B)^{2^n}}\right)}{T_t} - \left(\frac{-\ln\left(1-(1-x^B)^{2^n}\right)}{T_t}\right) \tag{A16}$$

Simplifying:

$$r_H^B = \frac{\ln(2^n x^B)}{T_t} \tag{A17}$$

Substituting for x^B:

$$r_H^B = \frac{\ln\left((2^n)\exp(-k_m g T_t)\right)}{T_t} \tag{A18}$$

Simplifying further:

$$r_H^B = \frac{\ln(2^n) - k_m g T_t}{T_t} \tag{A19}$$

$$r_H^B = \frac{\ln(2^n)}{T_t} - k_m g \tag{A20}$$

$$r_H^B = r_L^B \tag{A21}$$

In scenario B, we can see explicitly how consideration the births and deaths of colonies gives us exactly the same bottom line (fitness) as consideration of the births and deaths of lone cells.

Cell-level fitness, scenario C

In scenario C, the only way the cells differ from scenario A is their mortality rate. By substituting the scenario C mortality rate into the scenario A fitness equation we get:

$$r_L^C = \frac{\ln\left(\frac{c + k_g g t_s}{c}\right)}{T_t} - \frac{k_m g(\alpha + \beta)}{\alpha\left(\frac{c + k_g g t_s}{c}\right) + \beta} \tag{A22}$$

Colony-level fitness, scenario C

The colony-level generation time remains unchanged from the generation time in previous scenarios. To calculate the colony-level fecundity in scenario C, we begin by defining the probability that a cell will survive for one generation (x^C):

$$x^C = \exp\left(-\left(\frac{k_m g(\alpha + \beta)}{\alpha 2^{\left(\frac{c + k_g g t_s}{c}\right)} + \beta}\right) T_t\right) \tag{A23}$$

The rest of the logic for calculating colony-level fecundity remains the same as in scenario B. This gives us:

$$F_H^C = \frac{2^n x^C}{1 - (1 - x^C)^{2^n}} \tag{A24}$$

The colony-level mortality in scenario C is the same as in scenario B (Eqn. A15) except with the scenario B cell mortality rate replaced by the scenario C cell mortality rate:

$$M_H^C = \frac{-\ln\left(1 - (1 - x^C)^{2^n}\right)}{T_t} \tag{A25}$$

The colony-level fitness in scenario C is the same as in scenario B except with the scenario B cell mortality rate replaced by the scenario C cell mortality rate:

$$r_H^C = \frac{\ln(2^n x^C)}{T_t} \tag{A26}$$

The same steps (substituting and simplifying) as in scenario B can be followed to show:

$$r_H^C = r_L^C \tag{A27}$$

APPENDIX C. BODY SIZE, OFFSPRING NUMBER, AND GENERATION TIME IN VOLVOCINE ALGAE: REGULATION OF INTERRELATED TRAITS DURING A MAJOR EVOLUTIONARY TRANSITION

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Abstract

Volvocine algae (Volvocaceae and closely related colonial and unicellular algae) have an asexual cycle in which a number of cell divisions occur in rapid succession following a prolonged period of growth. In unicellular *Chlamydomonas reinhardtii*, the average number of rounds of cell division per cycle, $\bar{n}$, increases approximately linearly with increasing light intensity for cells grown in constant light. This reaction norm reflects regulation of cell number, a trait that is thought to be among the first to have changed during the unicellular-to-multicellular transition in volvocine algae. A widely accepted and empirically supported explanation for how and why *C. reinhardtii* cells achieve this pattern is lacking. We propose a model in which cells first grow for a constant time period during which divisions cannot occur. Based on the cell size after this time period, each cell then establishes a target volume and divisions are initiated when the target volume is reached. This model reconciles cell- and population-level observations of *C. reinhardtii* cell division. We also present an optimality analysis of the $\bar{n}$ reaction norm on light intensity. We focus on two largely endogenous factors: time needed to switch between growth and divisions and size-dependent cell growth. The combination of this cost and benefit can give a reaction norm in which optimal $\bar{n}$ increases as a function of increasing light intensity (as is seen in *C. reinhardtii*). Finally, we explore the way in which dependence of cell growth rate on colony size could favor lower $\bar{n}$ for a given light intensity in colonies compared to unicells. If this effect is indeed relevant to early-branching colonial volvocines, then n regulation in these simple colonies would be adaptive specifically at the group level. This suggests the possibility that group-level individuality in the sense of specifically group-level adaptedness emerged very early in the volvocine transition to multicellularity in spite of the fact that group-level individuality in the sense of indivisibility of the higher level emerged later.

Introduction

The spheroidal, photosynthetic colonies of the green alga *Volvox carteri* consist of a surface layer of cells connected by extracellular matrix, which also fills the interior of the colony. Two cell types can easily be distinguished in the asexually-reproducing colonies: larger gonidia, which produce the colonies of the next generation, and smaller somatic cells, whose flagellar beating propels the colony in its characteristic rolling motion. *Volvox* is an example of clear colony-level functional integration, an outcome which is a hallmark of multicellular-level natural selection. There is no doubt that *Volvox* cell-level morphology and behavior have been shaped by colony-level natural selection so as to function as a part of a larger whole. This may seem unremarkable until one considers that not so far back in the ancestors of *Volvox*, multicellular entities did not exist and cells functioned only as wholes, not as parts of wholes. How and why do former wholes become mere parts of a new kind of whole? This is a focal question of work on "major transitions" or "evolutionary transitions in individuality" (Buss 1987; Maynard Smith 1988; Maynard Smith and Szathmáry 1995; Michod 1999).

Volvox and its close colonial and unicellular relatives are particularly appealing subjects to study from this perspective because middle ground in the hierarchical level of functional integration (again, a hallmark outcome of natural selection at a particular level) is abundantly apparent. Between the bookends of *Volvox carteri* (in which the whole is the group of cells, i.e. the colony) and *Chlamydomonas reinhardtii* (in which the whole is the cell) exist a diversity of colonies with various grades of colonial-level functional integration (Figure 1). Colonies in the genera *Basichlamys, Tetrabaena*, *Gonium*, *Eudorina,* and *Pleodorina* show increasing grades of colony-level functional integration. Even the most unambiguous indication of higher-level functionality—reproductive and somatic division of labor—appears in grades, with some species

having cells that behave as soma facultatively. The living intermediate-form volvocine algae give hints about the historical changes that set a unicellular ancestor of *Volvox* on a path to multicellularity (Herron and Michod 2008). Volvocine algae help us to think concretely about how the unit of selection—and thus the hierarchical level of function—might have gradually shifted to the multicellular level.

After decades as a leader in the molecular genetics of development in *Volvox carteri*, David Kirk wrote a "Problems and Paradigms" paper (2005) in which he aimed to "outline twelve important ways in which the developmental repertoire of an ancestral unicell similar to modern *C. reinhardtii* was modified to produce … eventually *Volvox carteri* with its complete germ-soma division of labor." This "twelve-step program" has served as a foundation for thinking about how a series of small developmental changes can span the seemingly vast divide between an evolving population of cells and an evolving population of cell-groups/colonies (Herron and Michod 2008; Herron 2009; Herron et al. 2009; Nishii and Miller 2010; Hallmann 2011; Umen and Olson 2012). Kirk's twelve step framework is in need of further development in order for progress to be made in connecting particular historical changes to more fundamental levels-of-selection issues. In this work, we consider in more detail the changes in "genetic modulation of cell number" (Kirk 2005), a category which is thought to capture one of the earliest changes in the evolutionary branch that led from a unicellular ancestor to modern colonial volvocine algae (Herron and Michod 2008).

"Genetic modulation of cell number" relates to a particular aspect of the *Chlamydomonas* asexual cell cycle. The *Chlamydomonas* asexual cell cycle consists of a growth phase, in which the cells increase in volume, and a division phase, in which the cell undergoes a rapid series of n synchronous divisions without intervening growth (where n is an integer ≥ 1) (Harris 2008;

Figure 2A). G_1 refers to Gap 1, the phase during which cells grow, and S/M refers to a round of DNA synthesis (S) and mitosis (M). Mitosis quickly follows DNA synthesis and the gap between them (G_2) is negligible in *Chlamydomonas*, so synthesis and mitosis are treated together here. After an initial G_1-to-(S/M) transition, *Chlamydomonas* cells make $n-1$ additional, rapid (S/M)-to-(S/M) transitions with negligible intervening cell growth. To complete the cycle, the cells transition out of (S/M) phase. Newly divided cells enter G_1 and start to grow immediately if external conditions allow. If external conditions are not conducive to growth, the newly divided cells enter a resting state (G_0), with the start of growth (G_1) occurring as soon as conditions are conducive to growth. This kind of cycle is known as palintomy[1]. The number of offspring cells per G_1-$(S/M)_n$-G_1 cycle is 2^n, with n indicating the number of rounds of cell division per cycle. The number of offspring cells is the "cell number" referred to in Kirk's (2005) "genetic modulation of cell number", so this step is equivalent to changes in the internal regulation of n. Modulation of n is intimately tied to palintomy in unicells and takes on new significance in colonial life cycles. In the earliest-branching undifferentiated colonial volvocines, every colony derives from the products of mitosis of a single parental cell and every cell produces a colony of the next generation. This kind of colonial cycle is known as autocolony (Figure 2B). Regulation of n in a colonial volvocine species dictates the number of offspring colonies per cycle and also the number of cells per colony.

It is well-known that in both *Chlamydomonas* and the colonial volvocine algae, the number of rounds of division per cycle (n) is highly dependent on the quality of the external

[1] Palintomy, a type of multiple division, refers specifically to cycles in which a long period of growth alternates with a rapid sequence of binary divisions (Sleigh, 1989, p.95). Palintomy is characteristic of many chlorophytes (Šetlík and Zachleder 1984) and has been noted in other microbial eukaryotes. Examples of studies on regulation of multiple division in non-volvocine species include: Poyton and Branton (1972) (the green alga *Prototheca zopfii*) and Lam et al. (2001) (the dinoflagellate *Crypthecodinium cohnii*).

environment (Kirk 2005). Worse environmental conditions generally produce cells or colonies with lower n. Kirk (2005) commented that "in all of the multicellular forms there is an additional layer of genetic regulation imposed that results in different maximal values of n in different species." (Emphasis in original.) Indeed, in species descriptions or comparative analyses of colonial volvocine algae, maximum n value is often reported as a species-specific character (Šetlík and Zachleder 1984; Coleman 2012). The conceptual framework here is that species-specific genes or alleles determine the maximum n-value in colonial volvocine algae whereas environmental conditions can cause sub-maximal n-values in all species.

One problem with this approach is the impossibility of knowing whether a species' maximal n has been observed. Some as-yet-undiscovered environmental conditions could increase observed n further for any given species. More fundamentally, the focus on maximal n misses the point that the effects of "nature" and "nurture" can be more neatly separated (at least to a first approximation) by consideration of the entire reaction norm of a trait (Stearns 2000). That is, a genotype can be thought of as specifying what a colony's n value would be under a range of possible environmental conditions (Schlichting and Pigliucci 1998). Changes in the nature of this functional relationship constitute changes in the regulation of n. For example, if n increases linearly over a certain range of light intensities, then changes in the regulation of n could manifest as a change in the slope or intercept of n against light intensity. Given some constraints on light intensity/cell growth rate, the maximum n would also change, but this would be incidental to the underlying change in the overall n-versus-environment relationship. In this work, we take changes in the genetic (or internal) regulation of n to mean changes in genes that affect the relationship between n and an environmental parameter (i.e. the reaction norm of n on an environmental parameter).

C. reinhardtii n regulation: the pattern to be explained

At the molecular level, the explanation for how *C. reinhardtii* regulates n (and cell cycle progression more generally) involves the integration of many pathways, each conveying internal and/or external information, to ultimately affect gene expression. The repertoire of genes related to cell cycle regulation in *C. reinhardtii* is similar other eukaryotes such as higher plants and metazoans (Bisova et al. 2005). Umen and Olson (2012) suggested that the CDKG1 and CDKG2, cyclin-dependent kinases that are not found outside of volvocine algae, may be related to *Chlamydomonas* palintomy. Other relevant proteins that have been studied in volvocine algae include D-type cyclins and RB tumor suppressor-related proteins (RBRs; Umen and Olson 2012 and references therein). Overall, however, the current picture concerning the genetic basis for *Chlamydomonas* palintomy (as opposed to binary fission) as well as the genetic basis for regulated plasticity in palintomy (i.e. in n) is sparse. In this work, we are concerned with the way in which the cell assesses and responds to relevant factors at a phenomenological level. That is, we are concerned with the level of the behavior of the cell. We set aside the issue of how, at the molecular level, assessments and integration of relevant information might be accomplished by a cell.

Vítová et al. (2011b) presented data on the reaction norm of n on light intensity for cells grown in continuously diluted cultures under constant light[2] with CO_2 aeration. In Vítová et al.'s (2011b) Table 1, they reported the light intensity at which cells were grown, the population

[2] Some of the Vítová et al. (2011b) data are from cells grown in light-dark cycles, with the onset of the dark cycle set by the time at which a proportion of the cell population started to divide. Presumably the rest of the clonal population would have begun to divide soon thereafter even if the light were left on. Thus, their reports of cell cycle duration in light-dark condition reflect the endogenous "decision" of cells to begin divisions. For our purposes, these conditions are "constant light" because the properties of interest (i.e. the time and size of initiation of divisions) relate to the behavior of the cells prior to the light-dark transition. By contrast, when the light-dark cycle is set independently of the cell behavior, it is possible that the light-to-dark transition induces the initiation of divisions.

growth rate (calculated from the dilution rate necessary to maintain the population at a constant level), the average number of offspring cells per colony, and cell cycle duration (ending at the midpoint of daughter cell release). All of these were measured at the population level. These data show the basic pattern on which we focus here. Number of rounds of cell division per cycle, n, increases approximately linearly as light intensity increases (Figure 3; Appendix 1). Note that with a few reasonable assumptions, our relationship of interest (n versus light intensity) corresponds to the relationship between the base 2 logarithm of the final volume (i.e. volume at the initiation of divisions in constant light) and cell exponential growth rate (Figure 3).

How do cells achieve this pattern? Hypotheses concerning *C. reinhardtii* cell cycle regulation have long centered on the concepts of sizers and timers (Figure 4). An absolute sizer means that attainment of a particular size causes the next event to occur, whereas an absolute timer means that passage of a fixed time period (relative to some other internal or external event) causes the next event to occur. An absolute sizer or absolute timer produces particular relationships among final size, duration of growth, and growth rate (Figure 4). Note that if any two of the three (final size, duration of growth, and growth rate) are fixed, then the third is determined. For example, a cell cannot independently adjust its final size (n) and its duration of growth, given an environmentally-determined cell growth rate. That is, lifetime average body size, offspring number, and generation time are closely interrelated in *C. reinhardtii*.

Regulation of the initiation of divisions is closely linked to n regulation in *C. reinhardtii* because size at initiation of divisions determines n (see below). The initiation of divisions in *C. reinhardtii* clearly cannot be explained by an absolute sizer or an absolute timer alone. Neither size at initiation of divisions nor time of initiation of divisions (relative to the start of growth) is

constant in *C. reinhardtii* (Figure 4C). This has led to the proposal of various combinations of sizer and timer sub-periods within G_1 in order to explain the overall pattern.

In this work, we first review some of the most common current ideas concerning regulation of number of rounds of division per cycle in *C. reinhardtii*. We discuss relevant empirical work, especially from several recent studies (Matsumura et al. 2010; Vítová et al. 2011b). We propose a phenomenological model of *C. reinhardtii* n regulation that is consistent with the major empirical patterns described in the literature. Next, we consider how two factors, switching time and size-dependent growth, can affect optimal n regulation. Metabolic scaling theory supports a strong connection between body size and growth rate. Since the transition from palintomy to autocolony potentially involved a substantial increase in body size, understanding how size-dependent growth influences optimal n reaction norms is especially relevant to understanding the early changes in n regulation in the ancestors of colonial volvocine algae.

The standard model

Controversy concerning details notwithstanding, some major elements of what we call a "standard model" of *C. reinhardtii* regulation of initiation of division/n are often repeated (see Appendix 2). The first of these elements is uncontroversial: size-dependent cessation of divisions. It has long been recognized that the cessation of divisions (i.e. the (S/M)-to-(G_0 or G_1) transition) depends on the size of the parent cell at initiation of division (Harris 2009, p.82). This aspect of cell cycle regulation has been called the "mitotic sizer" (Bisova et al. 2005). The mitotic sizer is a threshold size that offspring cells must equal or exceed in order to divide again. Once offspring cells fall below this threshold size, a (S/M)-to-(G_0 or G_1) transition is triggered. This leads to at most a two-fold range in size of newly hatched cells, regardless of the size of the parent cell. The existence of a mitotic sizer is supported by the correlation between n and

parental cell size at initiation of division as well as by the observation that offspring cell volume is similar for cells produced by different-sized parents (Craigie and Cavalier-Smith 1982; John 1987; Matsumura et al. 2003b; Umen and Goodenough 2001).

A second element of the standard model concerns an important and recognizable benchmark in the *C. reinhardtii* cell cycle known as the Commitment Point (CP). The CP is defined as the point in the cell cycle after which a cell divides at least once even in the absence of light (Craigie and Cavalier-Smith 1982; Donnan and John 1983; McAteer et al. 1985; Spudich and Sager 1980). Pre-CP cells that are exposed to prolonged darkness enter a resting state; post-CP cells that are exposed to prolonged darkness divide and then the offspring cells enter a resting state. The CP is thought to be primarily determined by attainment of a critical size (Craigie and Cavalier-Smith 1982; John 1987; Matsumura et al. 2003b; Oldenhof et al. 2007; Spudich and Sager 1980; Umen and Goodenough 2001). After two-fold growth, cells typically pass the commitment point.

The final element of the standard model is an approximately constant timed delay of ~5–10 h between attainment of commitment and the initiation of division. Authors vary as to whether they present this element of the model as an explanation (i.e. cells initiate divisions because a time period since the CP has elapsed) or as a description (i.e. cells, for some unknown reason(s), appear to initiate divisions ~5−10 h after passing the CP). See Appendix 2. We use the phrase "standard model" to refer to the former idea: that a fixed time period measured from the CP explains the "decision" of cells to initiate divisions when light is constant.

We now determine n using the standard model. We assume cell volume grows exponentially at a rate r, so $V_{\mathrm{f}} = V_0 e^{r t_{\mathrm{grw}}}$ where V_{f} is the final volume of a parental cell after growth, V_0 is the initial volume of a parental cell, and t_{grw} is the amount of time that the cell

grows. In the standard model, a cell grows for the time needed to double in volume (i.e. for $\frac{\ln(2)}{r}$ hours) and then continues growing during a delay period, t_{dly}. So $t_{\mathrm{grw}} = \frac{\ln(2)}{r} + t_{\mathrm{dly}}$. The final volume of the parent is divided into 2^n equally-sized offspring such that offspring volume is greater than or equal to V_{min}. Fractional rounds of divisions cannot occur, so n is given by

$$n = \left\lfloor \log_2 \left(\frac{V_0 e^{r\left(\frac{\ln(2)}{r} + t_{\mathrm{dly}}\right)}}{V_{\mathrm{min}}} \right) \right\rfloor. \quad (1)$$

Note that the square brackets indicate a floor function. If $V_0 \approx V_{\mathrm{min}}$, then

$$n = \left\lfloor 1 + \frac{t_{\mathrm{dly}} r}{\ln(2)} \right\rfloor. \quad (2)$$

Here we see that by the standard model, n-value/final parental cell size in constant light is controlled internally by the duration of the delay period, t_{dly}, and externally by the growth conditions prevailing during the delay, r (Figure 5A). (The mitotic sizer is assumed constant as is the size at the commitment point.) The standard model basically proposes that the time/size of divisions is driven by a sizer (attainment of commitment after 2-fold growth) followed by a timer (constant delay period). Also see Appendix 2, Figure 5. On the face of it, this model fits well with the general pattern observed in *C. reinhardtii* cells exposed to constant light (Figure 6A). Faster-growing cells generally divide sooner and at a larger size compared to slower-growing cells. The standard model can account for this pattern because faster-growing cells traverse the first, pre-CP, part of the cycle faster (generally resulting in shorter time to divisions) and grow more during the second, post-CP, part of the cycle (resulting in larger size-at-division).

Problems with the standard model

The standard model proposes dividing the overall period of interest into two time periods: the start of growth to CP and CP to the start of divisions, with the first being controlled by an absolute sizer and the second being controlled by an absolute timer (Figure 5). While the first

assertation—that an absolute sizer controls the CP—is relatively well-supported, the second assertation—that an absolute timer controls the period between CP and divisions—is not.

At least two lines of evidence indicate that the period between CP and division may not be controlled by an absolute timer. First, recent single-cell level data of growth and division in constant light indicate that cells are dividing in response to attainment of a particular size, not in response to a particular time (Matsumura et al. 2003b; Matsumura et al. 2010). Based on the standard model, we would expect to see that the final size of a cell is not limited to be close to a value of $V_0 2^{\text{integer}}$ (Figure 7A). However, the single-cell level studies mentioned above generally found that cells in constant light grew to a size of $V_0 2^{\text{integer}}$. The distributions of final sizes over varying n values was not analyzed directly, though the results of these studies generally indicate that cells in constant light make the final decision to divide based on size (as is shown schematically in Figure 7B). Note that if cells divide in response to attainment of a target size, as in Figure 7B, an explanation for variation in target size is still needed. We return to this issue later.

Second, according to the standard model, the time between the CP and divisions is constant over changes in growth rate (Donnan and John 1983). Vítová et al. (2011b) measured post-commitment time and concluded that it is "more or less constant over a large range of growth rates". However, a two-fold range of average post-commitment times was reported (5 h to 10 h) for non-mutant cells assayed under different light intensities (i.e. cell growth rates). Such levels of variation in average post-commitment time could be compatable with the standard model. However, it is also possible that the underlying relationship between average post-commitment time and light level is not a horizonal line; if so, this could explain some of the large variation in average post-commitment time. Below, we consider the possibilty that the

underlying relationship average post-commitment time and light level is an ocsicllating function. Current empirical data does not clearly favor one possibility over the other.

In reference to their observation of a more or less constant post-commitment duration, Vítová et al. (2011b, p. 84) wrote "…we propose that, even in this case, no timer is required because the phase consists of a sequence of well-defined events (DNA replication, mitosis, cytokinesis), the duration of which is determined by the time required for the course of chemical reactions that receive their energy requirements from intracellular energy reserves." We think that this explanation falls within our phenomenological meaning of "absolute timer" (Figure 4), though Vítová et al. (2011b) presumably intended a more mechanistic meaning of "timer" in the above quote. The verbal model basically proposes a constant, light intensity-independent, time between the CP and the initiation of divisions, so (as described above) more analysis is needed to make a convincing case that the data support this model. Furthermore, the idea that the post-commitment duration is determined by the time required for the course of molecular events involved in initiation of divisions does not sit well with the fact that many cells, including close relatives of *Chlamydomonas*, divide by binary fission. If the delay between CP and division is simply the time needed for *Chlamydomonas* cells to initiate divisions, then why is this time so much longer in *Chlamydomonas* cells (which can undergo many more commitments during the delay) compared to binary fission cells (which essentially divide immediately after attaining two-fold growth)? The existence of binary fission cells indicates there are not hard and fast constraints making the delay period fall in the 5−10 h range.

The standard model and light-dark cycles

One variant of the standard model downplays the importance of the duration of the delay in regulating n by emphasizing the behavior of cells when exposed to a light-dark cycle (e.g.

Dillard et al. 2011; Olson et al. 2010). If the endogenous control of the delay period causes divisions to be timed during the night (i.e. if growth begins with the beginning of daylight and if the time of attainment of two-fold growth plus ~5−10 h is greater than daylength), then the total "extra" growth that occurs after the CP (which determines n) would simply be driven by daylength, an external factor. Thus, there would effectively be no internal regulation of n in *Chlamydomonas* (Figure 5C).

Some observations support the idea that parental size-at-division can be explained simply as the size of a parental cell when the external stimulus of a light-to-dark transition causes the G_1-to-(S/M) transition. Under standard culture conditions (growing cultures, sufficient light intensity, 12/12 light/dark cycle, 22-25°C), the rapid series of cell divisions consistently occur not long after the beginning of the dark period. Furthermore, when *C. reinhardtii* cells are grown heterotrophically in the dark, exposure to light delays the G_1-to-(S/M) transition (Münzner and Voigt 1992). Blue light exposure (which occurs more at midday in nature) can inhibit the G_1-to-(S/M) transition whereas red light exposure (which occurs more at dawn or dusk in nature) can release this inhibitory effect (Oldenhof et al. 2006). These results indicate that external light-to-dark transitions are a legitimate factor affecting the G_1-to-(S/M) transition. From this fact and the observation that cells in nature typically experience a day-night cycle, one may be tempted to infer that the duration of delay period is not likely to play a major role in regulating n in nature.

Ultimately, however, the idea that parental size-at-division is completely driven by external cues does not account for all the relevant behavior of *Chlamydomonas* cells. When cell cycle transitions are largely (or entirely) driven by an external stimulus (light/dark cycling), all cells in a culture will be at approximately the same stage in the cell cycle at any given time. Such cultures are said to be synchronous, and synchronous cultures of *Chlamydomonas* cells can be

attained by exposing cells to a light/dark cycle. However, the degree of synchrony is generally higher for cells kept under better growth conditions and is imperfect even under the best conditions (Lien and Knutsen 1979; Lemaire and Hours 1999; Griffiths 2010). In synchronous cultures, population increase is stepwise (with constant population during the day and a jump in population level during the night); by contrast, population level increases smoothly in asynchronous cultures of *Chlamydomonas*. This indicates that divisions are happening during the day (light period) in asynchronous cultures, so the external forcing of divisions by the light-dark cycle is not completely effective unless growth conditions are very favorable. Since conditions in nature are not always conducive to high cell growth rates, the level of synchrony of divisions (i.e. the effectiveness of external forcing) in nature is likely to be less than what is observed under favorable lab conditions. Conditions in nature are important for understanding the potential adaptive benefit of regulatory mechanisms, which is our focus here. Although conditions of continuous or near-continuous light are only experienced by high-latitude cells in nature, these conditions nonetheless give an important window into the behavior of internal cell cycle regulatory mechanisms. These mechanisms are probably expressed to varying degrees in other environments. Cells grown in continuous light undergo divisions, and this simple observation is enough to indicate that the light-to-dark transition is not necessary to induce the G_1-to-(S/M) transition. To understand changes in the regulation of n, we want to understand the internal rules that a cell uses to decide to divide; that the internal rules may sometimes be masked is not critical. We assume that these internal rules have an effect at least sometimes and thus have been subject to natural selection.

Growth rate-dependent target size (GR-TS) model, single cell-level

We now develop a model to try to accommodate the observation that growth to a target size rather than an absolute timer appears to be controlling the G_1-to-(S/M) transition (Matsumura et al. 2003b; Matsumura et al. 2010). If the end of growth is determined by a target size, then there has to be some way for the target size to increase with growth rate/light intensity. A size that increases with growth rate is the hallmark feature of an absolute timer (Figure 4), suggesting that an absolute timer followed by a sizer (Figure 5B) might be able to account for the major patterns in *C. reinhardtii* cell behavior. The proposed model would reconcile the single-cell-level observation of growth to a target size and the population-level observation of substantial variation in final sizes.

To follow up on this idea, we base our model on the assumption that there is a constant (growth rate-independent) time period during which the cell grows without dividing. We call this time t_{chk}. We further assume that the cell establishes a target final size based on its size after the constant time period of growth, i.e. based on $V(t_{\mathrm{chk}})$. Note that if the cell retains information about its initial size (or if initial size is invariant), then basing a target final size on $V(t_{\mathrm{chk}})$ is equivalent to basing a target final size on early growth rate. (The early-life exponential growth rate is given by $\frac{1}{t_{\mathrm{chk}}} \ln\left(\frac{V(t_{\mathrm{chk}})}{V_0}\right)$.) Hence, we call this model the growth rate-dependent target size (GR-TS) model. We start by assuming that the cell grows to the next-highest target size compared to its size at the end of the constant time period, $V(t_{\mathrm{chk}})$. A target size is given by $V_0 2^n$ where V_0 is the initial volume and n is an integer. Growth to a target size would help to explain why the amount of time growing is generally, but not always, smaller for faster-growing cells (i.e. faster-growing cells reach their target more quickly). The larger size of faster-growing cells would be explained by the early, constant time period (during which faster-growing cells

grow more). Finally, the smallest threshold size above which the cell still divides (the mitotic sizer) is still needed to translate between target final size and n value. Overall, the model consists of a time phase (the constant period of growth, after which size is evaluated), then two size phases (growth to a target size, which is determined by the previous phase, and division until a threshold size).

To start, we consider exponential cell growth, so cell volume, V, is given by

$$V(t) = V_0 e^{rt}. \tag{3}$$

See Table 1 for descriptions of symbols used. A constant, growth-rate-independent period of time (measured from the start of growth) elapses between the beginning of growth, $t = 0$, and the time at which the cell will check its size, $t = t_{\mathrm{chk}}$. The volume of the cell at the check time is given by $V(t_{\mathrm{chk}})$. The target volume of the cell is given by the next-highest volume compared to $V(t_{\mathrm{chk}})$ that will make n an integer. We assume complete conservation of volume and assume $V_0 = V_{\mathrm{min}}$ so

$$\frac{V_{\mathrm{f}}}{V_0} = 2^n, \tag{4}$$

where V_{f} indicates the final volume of the cell after all growth is complete. The assumption that $V_0 = V_{\mathrm{min}}$ is reasonable if cells in constant light are dividing in response to attainment of a threshold size. Note that as long as initial size, V_0, is constant, V_{f} completely defines n and vice versa. That is, lifetime average body size (where T is the length of the lifetime, $\frac{1}{T}\int_{t=0}^{T} V(t)$) and number of offspring $\left(\frac{V_{\mathrm{f}}}{V_0}\right)$ are closely interrelated traits.

The number of divisions per cycle is given by

$$n = \left\lceil \frac{r t_{\mathrm{chk}}}{\ln(2)} \right\rceil. \tag{5}$$

Note that the square brackets indicate a ceiling function. We see that in this model, the external environment affects n through the value of r whereas regulation of n-value by the cell can be achieved by genes that affect t_{chk}. Recall that t_{chk} determines the duration of the period of growth, after which the cell evaluates its size and commits to an n-value based on that size. The target final volume is given by

$$V_{\mathrm{tar}} = V_0 2^{\left\lceil \frac{r t_{\mathrm{chk}}}{\ln(2)} \right\rceil}. \tag{6}$$

We assume that once growth starts, it is not interrupted and that the cell grows until its final volume equals its target volume. The duration of growth is thus given by

$$t_{\mathrm{grw}} = \frac{\ln(2)}{r} \left\lceil \frac{r t_{\mathrm{chk}}}{\ln(2)} \right\rceil. \tag{7}$$

Because it is often asserted that the post-commitment time is approximately constant across growth rates (see Appendix 1), we asked what post-commitment time would look like as a function of growth rate for our model. Figure 7C shows that the GR-TS model implies an oscillating post-commitment time. For a given n, post-commitment time declines with increasing growth rate. However, there are also local increases of post-commitment time that correspond to increases in n-value. This model is broadly consistent with the high variation in average post-commitment time across light intensity treatments. The functional relationship between average post-commitment time and light intensity is an empirically-accessible relationship which would be substantially different according to the two models considered here. Another interesting implication of this model is that the least upper bound of post-CP duration is t_{chk}. Based on this observation and the data in Vítová et al. (2011b, their Table 1), about 10 h would be an estimate of *C. reinhardtii* t_{chk} based on post-commitment time alone. The single-cell level patterns that emerge from the check time model are plotted alongside population-level data in Figure 6B.

Growth-rate-dependent target size (GR-TS) model, cell population level

In order to compare our model to published data from populations of clones, we now develop explicit assumptions about cell-to-cell variation in such a population. In our model, the n-value of a cell is determined by its size at the check time, which in turn is determined by exponential growth of the cell,

$$V(t_{\text{chk}}) = V_0 e^{rt_{\text{chk}}}. \tag{8}$$

Cell volumes in *C. reinhardtii* are lognormally distributed (Garz et al. 2012), so we assume $V_0 e^{rt_{\text{chk}}}$ is lognormally distributed. Recall that V_0 is a constant in our model, allowing us to divide by V_0 and assume that $e^{rt_{\text{chk}}}$ is lognormally distributed. That is, rt_{chk} is normally distributed, i.e.

$$rt_{\text{chk}} \sim \mathcal{N}(\mu, \sigma^2), \tag{9}$$

where μ and σ^2 are the mean and variance (respectively) of rt_{chk}. We assume that all cells divide at least once and no cell undergoes more than 5 rounds of division. The relationship between n and rt_{chk} is thus given by

$$n = \begin{cases} i & \text{if } \ln\left(2^{i-1}\right) < rt_{\text{chk}} \leq \ln\left(2^{i}\right) \\ 5 & \text{if } \ln(2^4) < rt_{\text{chk}} \end{cases}. \tag{10}$$

We consider only cases in which $0 < rt_{\text{chk}}$ (i.e. size does not decrease over the early life of a cell). For example, a cell must grow at 2-least fold but no more than 4-fold by $t = t_{\text{chk}}$ in order to eventually undergo two rounds of division ($n = 2$) and give rise to four offspring cells.

The average n is given by

$$\bar{n} = \sum_{i=1}^{5} n_i \Pr(i), \tag{11}$$

Where Pr indicates the probability of a particular n vlaue. The probabilities of different n-values are given by

$$\Pr(n) = \begin{cases} \int_{\ln(2^{n-1})}^{\ln(2^{n})} \Psi(\theta) d\theta & \text{if } 1 \leq n < 5 \\ \int_{\ln(2^{n-1})}^{\infty} \Psi(\theta) d\theta & \text{if } n = 5 \end{cases} \quad (12)$$

where $\theta = r t_{\mathrm{chk}}$ and $\Psi(\theta)$ is the probability density function of θ.

In Figure 8A-C, $\bar{n}$ (the population mean of n) is plotted as a function of the growth rate of a cell, r, for a variety of t_{chk} values and for various levels of variability (CV) in $r t_{\mathrm{chk}}$. Note that, at the population level, if the *CV* (coefficient of variation, which is the standard deviation divided by the mean) of $r t_{\mathrm{chk}}$ is relatively high (e.g. 0.3), then $\bar{n}$ increases smoothly as a function of r except for at low growth rates. Because n cannot be less than 1 in this model, the $\bar{n}$ at low growth rates are higher than they otherwise would be. Note also that higher values of t_{chk} generally lead to a higher slope in the relationship between $\bar{n}$ and cell growth rate/light intensity.

Our model so far captures a major population-level pattern of *C. reinhardtii* cell cycle regulation: the approximately linear increase of $\bar{n}$ with increasing cell growth rate/light intensity. However, when we compare our model results to data from Vítová et al. (2011b, their Table 1), at least one inconsistency is noticeable. In the data, $\bar{n}$ rises from ~1 to ~2 at very low cell growth rates. The obvious way to make $\bar{n}$ higher at lower growth rates in our model is to increase t_{chk}. However, we see t_{chk} values greater than the shortest cell cycle duration observed in constant light, ~10 h, as unrealistic. If $t_{\mathrm{chk}} > 10$, then how were cell divisions initiated prior to t_{chk} in the shortest-generation time populations? Because of this limit on realistic values of t_{chk}, we sought another way to increase the $\bar{n}$ for a given cell growth rate and t_{chk} value.

Recall that in our model, cells that have grown between, for example, 2-fold and 4-fold when $t = t_{\mathrm{chk}}$ will go on to divide two times (i.e. $n = 2$ and $V_{\mathrm{tar}} = 2^2 V_0$). These boundaries and how they relate to target final growth were chosen arbitrarily, so it makes sense to introduce another parameter into the model that allows us to explore the effects of changing these

boundaries. We call this parameter f because it controls the relationship between fold-growth attained at t_{chk} and target final growth/n-value. The way in which f affects n is given by

$$n = \begin{cases} i & \text{if } \ln\left(2^{i-1}\right) - f < rt_{\mathrm{chk}} \leq \ln\left(2^{i}\right) - f \\ 5 & \text{if } \ln(2^4) - f < rt_{\mathrm{chk}} \end{cases}. \tag{13}$$

and

$$\Pr(n) = \begin{cases} \int_{\ln(2^{n-1})-f}^{\ln(2^{n})-f} \Psi(\theta) d\theta & \text{if } 1 \leq n < 5 \\ \int_{\ln(2^{n-1})-f}^{\infty} \Psi(\theta) d\theta & \text{if } n = 5 \end{cases}. \tag{14}$$

See Figure 8 and Figure 9 for an example of the effect of f on $\bar{n}$.

With the model that incorporates f, we see that the model matches the data relatively well for $f = 0.5$, $t_{\mathrm{chk}} = 6$, and $CV = 0.3$ (Figure 6C). Of course it is possible that other combinations of these three parameters match the data from Vítová et al. (2011b) equally well. For example, since the post-commitment time data gave an estimate $t_{\mathrm{chk}} \approx 10$, it could be useful to search for values of f and CV that give a good match to the data when $t_{\mathrm{chk}} = 10$. However, the inferring values for the model parameters based on empirical evidence is not critical for our purposes here. The important point is only that the GR-TS model can be consistent with the major pattern in *C. reinhardtii* cell cycle regulation: an increasing, approximately linear reaction norm of $\bar{n}$ on growth rate/ light intensity. Furthermore, the GR-TS model offers a potential (though untested) explanation for the wide variation in post-CP times (Vítová et al. 2011b) and the sizer-like behavior described in recent cell-level studies (Matsumura et al. 2003b; Matsumura et al. 2010) (Figure 7). Data collected for the purpose of comparing the two models would be necessary to give a more definitive comparison.

Our model explicitly posits two endogenously-controlled parameters that could affect the reaction norm of $\bar{n}$ on growth rate/light intensity: t_{chk} and f. Whether or not these two parameters can actually vary independently is unknown and depends on the nature of the genetic basis for these traits. This model suggests a different concept from the one articulated by Kirk (2005), who states that colonial species have an "additional layer of genetic regulation imposed that results in different maximal values of n in different species." By our proposed model, *C. reinhardtii* cells do control their maximal cell number (in constant light) through genes (and/or epigenetic factors) that cause specific values of f and t_{chk}, though our model suggests that maximal n value has no special significance compared to sub-maximal values. Control over cell number is not restricted to colonial species and thus hypothesizing an "additional layer of genetic regulation" in colonial species seems superfluous. Herron and Michod (2008) also noted that the "additional layer of genetic regulation" hypothesis is only one of several possibilities in need of further investigation. The way that *C. reinhardtii* regulates n could well be the same way that undifferentiated colonial volvocines control their cell number, albeit with different endogenously-determined values for the parameters f and t_{chk}. That is, rather than an "additional layer" of genetic control, changes in n regulation in colonial species might have required minor changes in an already-existing regulatory framework. Strains or mutants that differ in these parameters could be identified by observing the entire reaction norm (not just maximum n) of $\bar{n}$ on growth rate/light intensity.

Although Kirk (2005) explicitly proposes that colonial species have an "additional layer of genetic regulation" of cell number control compared to *C. reinhardtii*, his discussion of *mat3* mutants suggests that he also recognized the possibility of much more continuity in the genetic basis of cell number regulation in unicellular and multicellular volvocines. Citing personal

communication from James Umen, Kirk (2005) mentioned the hypothesis that "increases in cell number during volvocacean evolution have involved changes in the *mat3* orthologue that increased the threshold size required to initiate division, with no concomitant increase in the size of daughter cells at which division would stop." Mutants of *mat3* have substantially different behavior with respect to the size at which cells undergo commitment and the size at which cells stop dividing (Umen and Goodenough 2001). The above hypothesis would work well if divisions are initiated following a constant post-commitment delay because in that case, larger commitment size would lead to larger size at division, which leads to larger n. However, the proposed model suggests no direct connection between commitment size and size at division. Therefore, if the proposed model is supported by future empirical work, the above hypothesis would seem less tenable as a general explanation of the genetic basis for n regulation in volvocine algae. However, we did not directly explore the effects of allowing variation in initial size, the other major phenotype (besides commitment size) that is changed in *mat3* mutants. Again, our purpose here was to focus on the level of cell behavior (rather than genes underpinning those behaviors). It is clear, however, that advances at the two levels of analysis could complement each other.

Finally, note that the proposed model is agnostic among several possible mechanisms that the cell could use to accomplish n regulation. The model was described in terms of cells that grow for a constant time, evaluate their size at that time, then grow to a target size which depends on their size after the constant time. However, the model could equally well be described in terms of cells that divide after a growth rate-dependent time or at a growth rate-dependent size (Figure 6C, top and middle panel). Some evidence concerning how cells could monitor or control their growth rate and link this information to cell cycle progression is known

in other organisms (Ferrezuelo et al. 2012; Orij et al. 2012), so the latter kinds of interpretations might be closer to the underlying mechanisms. Nevertheless, at the level of cell behavior, deciding to divide based on a growth rate-dependent target size versus based on a timer-then-sizer are just different ways of describing the proposed model. Each of these possible descriptions of the proposed model contrasts sharply with the standard model, which can be thought of as a sizer-then-timer model.

Optimal reaction norm of n

If the reaction norm of $\bar{n}$ on growth rate/light intensity is endogenously controlled, this raises the question of why a particular $\bar{n}$ reaction norm is adaptive. Asking this question with respect to unicellular *C. reinhardtii* sets the stage for an analysis of why changes in this trait happened so early in the lineages leading to multicellular volvocine algae. That is, if an initial "step" towards multicellularity was a change in $\bar{n}$ reaction norm, we would like to know whether and why the ancestral reaction norm was adaptive as well as whether—at what hierarchical level—the change was adaptive.

We now investigate the cell number reaction norm that optimizes fitness. For simplicity, we consider a population of cells with no variation in n value or interdivision time (T). The number of cells in the population at time t is given by $x(t)$, and each cell divides into 2^n offspring after a time interval T. We assume the population is growing exponentially (i.e. it is large and asynchronously dividing). Therefore,

$$x(t) = x_0 2^{\alpha t}. \tag{15}$$

Because T is the time between divisions and we assume all offspring survive,

$$x(t+T) = x(t)2^n. \tag{16}$$

Using Eqn. (15) and Eqn. (16) and solving for α gives $\alpha = \frac{n}{T}$. Therefore, $x(t) = x_0 2^{\left(\frac{nt}{T}\right)}$. Re-writing this equation to show the intrinsic rate of population increase gives

$$x(t) = x_0 e^{\left(\frac{\ln(2^n)}{T}\right)t}. \tag{17}$$

The intrinsic rate of increase of the population, r_{pop}, is given by

$$r_{\text{pop}} = \frac{\ln(2^n)}{T}. \tag{18}$$

We assume that there are negligible density- and frequency-dependent effects, so the exponential growth rate of a population of clones, r_{pop}, is an appropriate proxy for evolutionary fitness (see Roff 2010, p. 4-5 for discussion).

The empirical pattern for the reaction norm of n on cell growth rate/light intensity is approximately an increasing straight line. In the previous section, we saw that particular values of t_{chk} and f, parameters assumed to capture endogenous characteristics of the cells, can create this pattern. Here, we focus on the pattern itself and its potential significance for fitness and do not consider explicitly how a cell achieves the pattern. The empirical pattern is based on $\bar{n}$, the average n taken over a population of cells. However, population growth rate (and thus fitness) is a function of the average n taken over the cells that are dividing at any given time. These two values are different when variation in interdivision time or n is substantial (Painter and Marr 1967). In this section, we avoid this issue by assuming no variation or very little variation in n. We still allow n to take on non-integer values, however, which is realistic only for average n in a variable population. Future work could explore the implications of explicitly considering population growth in a population with substantial variation of n values.

We now ask what the optimal n is as a function of light intensity (i.e. resource input), given different assumptions about how cells grow and divide. Throughout this section we

continue to consider the behavior of cells in constant light only. As noted above, the internal "rules" that lead to division at a particular size and time during a constant light period are important traits even if they are sometimes masked by division in response to an external light-to-dark transition.

Note that the fitness formula $r_{\mathrm{pop}} = \left(\frac{1}{T}\right) n \ln(2)$ reveals a fundamental trade-off. Cells would have higher fitness by increasing both $\left(\frac{1}{T}\right)$ and n. This is shown in Figure 10A, where red indicates highest fitness and gray lines show combinations of $\left(\frac{1}{T}\right)$ and n that give equivalent fitness. In volvocine algae (as well as many other organisms), final size is closely linked to the number of offspring (2^n). Because it takes a longer time to grow to a larger final size (all else equal), the fitness components T and n cannot be adjusted independently by the cell. Rather, for particular conditions, T is a particular function of n (or vice versa). Here we assume that $T = t_{\mathrm{grw}} + t_{\mathrm{div}} n + t_{\mathrm{oth}}$. That is, between the end of one round of divisions and the end of the next round of divisions, the cell spends t_{grw} hours growing, $t_{\mathrm{div}} n$ hours dividing (while not growing), and t_{oth} hours not growing or dividing. The t_{oth} term includes time needed to switch between growth and division (assuming a cell cannot grow while switching and dividing). We first consider the case in which cell growth is exponential $\left(t_{\mathrm{grw}} = \left(\frac{1}{r}\right) n \ln(2)\right)$ and there is no fixed per-generation switching time ($t_{\mathrm{oth}} = 0$ for all cells). In this case, $\frac{1}{T} = \left(\left(\frac{1}{r}\right) n \ln(2) + t_{\mathrm{div}} n\right)^{-1}$. We assume that cell exponential growth rate increases linearly with light level. Specifically, using data described in Appendix 1, we assume that $r = c_1 Q + c_2$ where Q is the light intensity in μmol photons m^{-2} s^{-1}. We also assume t_{div} is a constant. Therefore, in this case, the functional relationship between $\frac{1}{T}$ and n is given by $\frac{1}{T} = k n^{-1}$ where k is a constant at each light level

$\left(k = \frac{c_1 Q + c_2}{\ln(2) + (c_1 Q + c_2)t_{\text{div}}}\right)$. The white lines in Figure 10B show $\frac{1}{T}$ as a function of n for several different light levels (corresponding to different r levels). These lines show how our assumptions (light-dependent exponential cell growth, $t_{\text{div}} = \text{constant}$, $t_{\text{fix}} = 0$) constrain the combinations $\frac{1}{T}$ and n that a cell can achieve in a particular environment. Note that in this case, all of the combinations that a cell can achieve in a given environment give rise to the same fitness values. That is, the white lines in Figure 10B follow the lines of equal fitness. Algebraically, recall that $r_{\text{pop}} = \left(\frac{1}{T}\right) n \ln(2)$ and $\frac{1}{T} = kn^{-1}$, so in this scenario, $r_{\text{pop}} = k \ln(2)$. (When $t_{\text{div}} = 0$, fitness is simply given by the exponential cell growth rate, which we assumed here depends only on light level.) For a given environment (fixed r), the cell would have the same fitness if it divides into few offspring more frequently as it would if it divides into many offspring less frequently. The cost of longer generation time/higher n (i.e. slower cycling of generations) is exactly offset by the benefit of longer generation time/higher n (i.e. higher number of offspring per generation). See the first row in Table 2 and Appendix 3.

When a positive switching time($t_{\text{oth}} > 0$) is incorporated into the interdivision time and cell growth is still exponential, there is a cost only to lower n values. Each generation, the cell must go through a fixed time period during which neither growth nor divisions occur. By switching between growth and division less often, higher-n clonal populations experience this wasted time less often, and thus have higher population growth rates. For all light intensities, the optimal n in this case is simply the maximal value that n can have. Fitness increases with increasing n at all light intensities/cell growth rates. See Figure 10C. See second row in Table 2 and Appendix 3.

The category of switching cost (rather than switching time *per se*, which is modeled here) encompasses many of the ideas related to why *Chlamydomonas* evolved palintomy (n sometimes greater than 1) from ancestors that reproduced by binary fission (n always equal to 1). Palintomy in the close relatives of *Chlamydomonas* is thought to be an adaptation that is coupled to the presence of a rigid cell wall (Bell and Koufopanou 1991; Kirk 1998, p.102). In naked or scaled green flagellates (which lack a rigid cell wall), the basal bodies remain attached to the flagella while also serving as centrioles at the spindle poles during cell division (Kirk 1998, p. 98 and references therein). Thus, in scaly flagellates, the flagella are functional during cell division. By contrast, in *Chlamydomonas* and its relatives with a coherent cell wall, the flagella are not free to move into the position needed for the basal bodies to function in cell division. This limitation has been called the "flagellation constraint" (Koufopanou 1994). In *C. reinhardtii*, which are thought to live primarily in wet soil (as opposed to lakes or ponds), cells resorb their flagella prior to division. They also add a sticky substance to their outer surface, presumably in order to attach to a substrate while they are temporarily immotile. Offspring cells form flagella anew and are motile immediately upon hatching. If having closely spaced periods of immotility is more costly than having widely spaced (perhaps longer) periods of immotility, then palintomy could be favored. This would be the result of a kind of switching cost[3]. Kirk (1998, p. 102) suggests this explanation. A cell wall-related switching cost is a reasonable idea for why selection might favor higher n (palintomy) specifically in rigid-walled species, though a switching cost alone (without explicit benefits of lower n) does not explain the dependence of n on cell growth rate in constant

[3] The "switching cost" of a period of immotility apparently does not apply to many of the small colonial volvocines. In these species, the flagella are not resorbed prior to cell division. Rather, the flagella become detached from the basal bodies as the basal bodies take up their role in cell division. The flagella can still function without the basal bodies for a limited time, allowing for motility in dividing colonies as a long as divisions do not take too long (Kirk 1998, pp. 99-101 and references therein).

light (Table 2, row 2 and Appendix 3). Another potential switching cost relates to production of cell wall material (Bell and Koufopanou 1991). In the scaly flagellates, the outer boundary of the parental cell becomes the outer boundary of the offspring as the cell divides by binary fission. By contrast, parental cell wall material is discarded each generation in *Chlamydomonas*. This loss of biological material could be another type of rigid-wall-specific switching cost (Bell and Koufopanou 1991).

We now consider factors that could favor lower n values. One possibility is that if mortality occurs with equal probability to any cell at any given time, a cell is better off dividing as soon as it is able to. Chance extinction of the entire lineage is less likely the more individual descendants there are at any given time. We do not consider this effect further here.

Another fundamental factor that could favor lower n values is if the cell relative growth rate is a decreasing function of size. Lower n entails smaller average size of a cell over its lifetime compared to higher n. (See Rading et al. 2011 for analysis of stationary size distributions of populations with varying n.) Cell growth fuels population growth and therefore fitness. If cell growth rate is a decreasing function of cell size, then, all else equal, higher-n cells (by virtue of their larger lifetime average size) would have lower fitness.

The near-universal decline in relative growth rate with increasing organism size is a focal pattern in metabolic scaling theory, so we briefly describe its relevance here. West, Brown, and Enquist (2001) derived a general growth function from metabolic scaling assumptions and found that the resulting sigmoidal growth trajectory fits the growth pattern of a variety of organisms, including indeterminate growers. The West, Brown, and Enquist (2001) model captures how size-dependent relative growth rates can emerge because “the energetic cost of maintaining existing biomass increases more rapidly than the energetic capacity to synthesize new biomass”.

The West, Brown, and Enquist (2001) model is not directly applicable to *C. reinhardtii* growth because they take the cell as the fundamental unit, and thus restrict their focus to multicellular organisms. They also assume that organism growth occurs through the creation of new cells, which is not the case for colonial volvocine algae (in which organism growth occurs through an increase in the size of a fixed number of cells). Nevertheless, the fundamental ideas underpinning the West, Brown, Enquist (2001) model do not hinge on these details. The basic insight that the scaling of metabolic rate on size can have a large impact on growth trajectories is likely to apply to both unicellular and colonial volvocine algae.

Metabolic scaling can generally be described by the relationship $B = B_0 m^{\gamma}$, where B is the metabolic rate of an organism, B_0 is a taxon-specific constant, m is the mass of the organism, and γ is the metabolic scaling exponent. The value of γ can have a large impact on the functional form of growth because it determines whether the ability to synthesize new biomass keeps up with the cost of maintaining existing biomass as biomass increases. For example, if the metabolic scaling exponent is 1 (as has been suggested for some microorganims) (DeLong et al. 2010), then we would generally expect growth to be exponential, not sigmoidal. With respect to our optimality analysis of n, exponential growth means no growth-related cost to higher n values. However, if the metabolic scaling exponent is $3/4$ under optimal conditions and declines as resources become limiting (as has been suggested in phytoplankton) (Finkel et al. 2004), then the sigmoidal form of the growth trajectories would impose a substantial, resource-dependent cost of higher n values. Metabolic scaling theory has received relatively less application to microorganisms compared to larger organisms (Kearney and White 2012) and the picture concerning how metabolic scaling is likely to influence the growth trajectory across light intensities in *C. reinhardtii* is still developing.

Here, we model *C. reinhardtii* cell growth using a standard logistic curve. This function has the disadvantage of not being based on first-principle ideas concerning how growth occurs. However, our interest is in growth curves that change as a function of environmental input (light), and a first-principles analysis that would capture this dependence would be too complicated to fall within the scope of this work. See Beardall et al. (2009), Finkel et al. (2009), and Finkel and Irwin (2000) for work addressing some of the issues relevant to the effects of changing light levels on growth in different-sized microalgae. See Paine et al. (2012) for a description of commonly used growth functions. The standard logistic curve has the advantage that it captures the sigmoidal form of growth (i.e. a similar form to the trajectory that arises when $\gamma < 1$) and can be fit to growth data from *C. reinhardtii* grown under different light intensities. (We used data that was reported in Vítová et al., 2011b; see Appendix 1). From these fitted models, a reasonable phenomenological picture of how *C. reinhardtii* cell growth changes with light intensity can be incorporated into our analysis of optimal n reaction norms. (Note, however, that these data do not allow for development of understanding how cell size and light intensity might separately influence cell growth. Rather, these two factors are highly correlated in the Vítová et al. (2011b) data.) Future work could address the way in which specific limiting factors give rise to growth trajectories that depend on light intensity in *C. reinhardtii.* Future work could also explore the generality of our conclusions for different growth trajectories.

We assume that cell volume at time t is given by

$$V(t) = \frac{V_0 e^{\rho t}}{1 - \frac{V_0}{V_\infty} + \left(\frac{V_0}{V_\infty}\right) e^{\rho t}} \tag{19}$$

where V_0 is initial volume, V_∞ is the asymptotic volume as $t \to \infty$, and ρ is the exponential growth rate of cell growth if $V_\infty \to \infty$. The time spent growing is given by

$$t_{\mathrm{grw}} = \left(\frac{1}{\rho}\right) n \ln(2) + \left(\frac{1}{\rho}\right) \ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right). \quad (20)$$

Recall that with exponential cell growth, $t_{\mathrm{grw}} = \left(\frac{1}{r}\right) n \ln(2)$. The first term in Eqn. (20) is analogous to the exponential cell growth case. This term captures how growing time increases with increasing n simply because it takes longer to grow to a larger size, even when relative growth rate is constant. The second term captures the fact that when n increases, the growing time increases even more than we would expect if growth just continued at the same pace for a longer time. At very high V_∞, the second term in Eqn. (20) is negligible. In this case, a cell would grow for $\left(\frac{1}{\rho}\right) n \ln(2)$ hours in order to reach its target size, and lifetime average growth rate would be independent of n. However, if the second term is non-negligible (because of lower V_∞), the cell would grow for an additional $\left(\frac{1}{\rho}\right) \ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right)$ hours in order to reach its maximum size, and lifetime average growth rate would decrease with increasing n. See Appendix 3.

When we considered exponential cell growth, we assumed that exponential cell growth rate (r) increased linearly with light intensity, allowing us to work in terms of the reaction norm of n on growth rate rather than light intensity (Figure 3; Table 2, row 2). Here, we assume that both ρ and V_∞ increase with light intensity. Specifically, we assume $V_\infty = c_3 e^{c_4 Q}$ and $\rho = r = c_1 Q + c_2$where Q is the light intensity in μmol photons m^{-2} s^{-1} and $c_1, c_2, c_3,$ and c_4 are constants. See Appendix 1.

In the sigmoidal growth case, fitness is given by

$$r_{\mathrm{pop}} = \left(\frac{1}{\rho} + \frac{1}{n\,\rho\,\ln(2)} \ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right) + \frac{t_{\mathrm{div}}}{\ln(2)} + \frac{t_{\mathrm{oth}}}{n \ln(2)}\right)^{-1}. \quad (21)$$

When there is no cost to higher n because $t_{\mathrm{oth}} = 0$, the optimal n is simply the minimal n value for all light intensities. That is, $\frac{1}{n\,\rho\,\ln(2)} \ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right)$ is an increasing function of n for reasonable

V_0, V_∞, and ρ values. See Figure 10D, Table 2, row 3 and Appendix 3. This result is intuitive; the growth trajectory creates a cost to higher n values and there is no benefit to higher n, so optimal n is always as low as possible.

When a cost (sigmoidal growth trajectory) and a benefit (positive switching time) of higher n are considered together, we can recover a pattern in optimal n reaction norm that is similar to the observed *C. reinhardtii* n reaction norm. The cost and benefit of higher n are affected differently by changing light levels, so rather than an intermediate optimum n that is constant across light intensities, we get an increase in optimal n as light intensity increases (Table 2, row 4; Figure 10; Figure 11).

Finally, we consider how our model of optimal n reaction norm would be affected when the simplest colonies first evolve from unicellular ancestors. When the ancestral unicellular asexual cycle is palintomy, mutation(s) that simply cause offspring cells to adhere for some period of time (less than the time in between rounds of division) can create an undifferentiated colonial species with an autocolony life cycle (Figure 2). We do not consider the reason(s) for such a change here. Instead we ask, given that a simple autocolony life cycle has evolved from a unicellular palintomic life cycle, are the costs and benefits of n (given particular external conditions) likely to be affected? Note that the life cycle of a cell within a colony appears to be very similar before and after such a change. In both the unicellular and undifferentiated colonial species, a cell grows from V_0 to $2^n V_0$ and then divides into 2^n offspring cells. Note also that we can assume negligible genetic variation within small colonies, so cell fitness and colony fitness are optimized by the same trait values (see Shelton and Michod *In press*).

We already have a sigmoidal growth model for a single cell, and we have seen how this growth trajectory can affect optimal n. Now we are faced with the question of how to extend this

cell growth model to the growth of a colony. One possibility is that cells within a colony (at a given light intensity) grow in exactly the same way as unicells. We call this an in-name-only colony because the colony has no relevant effects. We can model the growth of an in-name-only colony by simply assuming that the size of the colony is 2^n times the size of a cell at every time point. Where V^{C1} is the volume of an in-name-only colony, we get

$$V^{C1}(t) = 2^n V(t) = \frac{2^n V_0 e^{\rho t}}{1-\frac{V_0}{V_\infty}+\left(\frac{V_0}{V_\infty}\right)e^{\rho t}}. \quad (22)$$

Another possibility is that the volume of contiguous biological material controls the slowing of growth at increased sizes. Note that if the "body size" relevant to growth is the colony size (rather than the cell size), then there is a drastic difference in size between palintomy and autocolony (Figure 2). In that case, a colony with 2^n cells, each of initial volume V_0, grows exactly as if the colony were just one giant cell with initial volume $2^n V_0$. Here it is the cells, rather than the colony, that have no relevant effects on overall biovolume growth. Where V^{C2} is the volume of a giant-cell-like colony, we replace V_0 with $2^n V_0$ in Eqn. (19) and get

$$V^{C2}(t) = \frac{2^n V_0 e^{\rho t}}{1-\frac{2^n V_0}{V_\infty}+\left(\frac{2^n V_0}{V_\infty}\right)e^{\rho t}}. \quad (23)$$

This gives us two different models of the growth of a cell within a colony,

$$V_1(t) = \frac{V_0 e^{\rho t}}{1-\frac{V_0}{V_\infty}+\left(\frac{V_0}{V_\infty}\right)e^{\rho t}} \quad (24)$$

or

$$V_2(t) = \frac{V_0 e^{\rho t}}{1-\frac{V_0}{V_{\infty/2^n}}+\left(\frac{V_0}{V_{\infty/2^n}}\right)e^{\rho t}}. \quad (25)$$

We assume all cells within the colony are equivalent. For a cell within the in-name-only colony, cell growth is the same as unicellular growth (i.e. Eqn. (19) and (24) are the same). In the second case, the colony as a whole grows to V_∞ as $t \to \infty$ (just as a giant cell would). For a cell within

such a giant-cell-like colony, its asymptotic size is restricted to be $\frac{1}{2^n}$ of the asymptotic size of the colony (V_∞). One option for a general equation for the growth of a cell within a colony is as follows,

$$V_g(t) = \frac{V_0 e^{\rho t}}{1 - \frac{V_0}{V_\infty/(a2^n + 1 - a)} + \left(\frac{V_0}{V_\infty/(a2^n + 1 - a)}\right) e^{\rho t}} \quad (26)$$

with $0 \leq a \leq 1$. When $a = 0$, we recover the growth trajectory of a cell within an in-name-only colony (equivalently, a unicell). When $a = 1$, we get the growth trajectory of a cell within a giant-cell-like colony. We think that the first colonial volvocine algae were likely to fall in between these two possibilities. That is, both cell size and colony size would be relevant to the growth trajectory of a focal cell. However, this hypothesis is in need of empirical testing. Surprisingly few empirical studies have addressed this kind of question about cell growth. Also, we note that it is possible that cells within a colony actually could grow better than they do on their own (Kirk 1998, pp. 56–67). However, we see this as unlikely to apply to the first, simplest colonies. The growth advantages that apparently lead to high gonidial growth rates (mixing of colony boundary layer by somatic cells, storage of phosphorous in the extracellular matrix) would not apply to early-branching colonials that lack somatic cells and substantial extracellular matrix.

Using this growth trajectory, we can again examine the optimal n reaction norm. Higher values of a correspond to a larger influence of colony size on the asymptotic size of a cell. Cell asymptotic size limits the possible values of n because $n \leq \log_2\left(\frac{V_\infty}{V_0}\right)$. Increasing a (i.e. increasing the effect of colony size on the growth of the cell) leads to fitness decreasing. This effect is stronger for larger n. (Compare the same line/environment in panels E and F of Figure 10.) The upshot is that higher a values lead to lower optimal n values, especially at higher light

levels (Figure 11). Lower "maximum" n (i.e. n under high light/favorable growth conditions) is the general pattern that has been described in the earliest-branching colonial volvocines compared to unicellular relatives). Although the robustness of this effect and its support from empirical data are yet to be tested, this study nonetheless indicates a possible adaptive explanation for lower maximum n in early-branching colonial volvocines. If there is a negative effect of colony-level size on cell lifetime average relative growth rate in the simplest volvocine colonies, then this is an additional cost of higher n that would have appeared concurrently with the evolution of the first autocolony life cycles. The near universal decline of relative growth rate with organism size indicates that such a relationship could well be at play, even as a new kind of organism first emerges. One could even imagine a scenario in which a detrimental stickiness mutation first created an autocolony life cycle from a palintomic one. Against this background, changes in n regulation that reduced n (in a given environment) would be a compensatory change, partially mitigating the negative effects of neighbor cells on focal cell growth by keeping group size small. Such a change could stem from minor changes in already-existing n regulatory networks since n value has important fitness consequences even in a unicellular species. Of course this is speculation only, but it is worth noting that possibilities of this sort are not obvious from considering shifts in the levels-of-selection in the abstract.

Here we have considered only one cost of higher n (sigmoidal growth) and one benefit of higher n (switching time). Of course, many other fitness-affecting factors are likely to change with body size as well. Notably, organisms with different body sizes face different predators, parasites, etc. and differ in their abilities to utilize resources that are unevenly distributed spatially or temporally at a particular scale. We chose to focus on switching costs and the growth trajectory because these two fitness-affecting factors are largely endogenous to the focal

organism. Thus, they are likely important in all ecological settings. Also, more information is available about growth and division in isolated *C. reinhardtii* cells than about the ecology of *C. reinhardtii*.

Conclusions

In constant light, an asexual *Chlamydomonas* cell grows for a time, then stops growing and divides its final body mass into equally-sized offspring which again begin growing. This simple life cycle has substantial implications for the interrelations between three major life history traits: body size, offspring number, and generation time. We have explored *Chlamydomonas* cell behavior with an eye towards understanding both how and why cells make the decision to switch from growth to division at a particular time and size. The motive for this focus is to set the stage for further investigation of how and why a unicellular life cycle can become a mere part of a group life cycle. When and why do unicellular traits—lifetime average cell size, number of cell divisions, cell generation time—become mere parts of a life history that is adaptive at the group level?

Kirk (2005) suggested that genetic control of cell number was a "step" along the way towards full multicellular-level individuality and Herron and Michod (2008) inferred that it must have been a very early step. Our analysis adds considerable depth to the sketch of "genetic control of cell number" offered by Kirk (2005). By modeling growth explicitly, we see that offspring number (i.e. cell number) is tightly coupled to lifetime body size and generation time in *C. reinhardtii*. The potential influence of the light-dark cycle on division number notwithstanding, it is clear that this suite of traits reflects important life history decisions of *C. reinhardtii* cells. As such, we would expect considerable regulatory control over division number in *C. reinhardtii*. The effects of this regulatory control can be assayed by observing the norm of

reaction of n against an environmental parameter; narrow focus on maximum n does not seem necessary or helpful.

We developed a model (the growth rate-dependent target size model) of how *C. reinhardtii* cells make the decision to divide in constant light. A major strength of our model is that it can reconcile single-cell level observations of growth to a target size and population-level observations of division at diverse final sizes. Another strength is the explicit connections between endogenous properties (t_{chk}, f) and cell number regulation, conceptualized as the entire norm of reaction of n on light intensity (not just maximum n). That is, this model suggest what cell behavioral properties might have changed during the "control of cell number" step. The proposed model is far from the final word on the subject, however, and the data needed to support or refute it are clear (Figure 7).

We also began to explore how the optimal n reaction norm might have changed as an autocolony cycle replaced a palintomic one. A thorough analysis of costs and benefits of changes in n was beyond the scope of this work, but the cost and benefit we considered were illuminating nonetheless. We found that the combination of a switching time (conceptually similar to switching costs often discussed in relation to *C. reinhardtii*) and size-dependent (sigmoidal) growth leads to an increasing straight line for an optimal reaction norm of n on light intensity. Against this backdrop, the extent to which the rudimentary properties of a colony (i.e. the number of cells in it) affect the growth of a cell emerged as a potent, and little-studied, factor relevant to understanding the optimal behavior of a cell within a colony. The results from considering detrimental effects of colony size on cell growth rate hint that the drop in n observed in early-branching colonial volvocines might be adaptive. However, understanding the earliest-branching volvocine colonies as well-adapted evolutionary individuals raises more questions.

How can cell traits that are beneficial because of the group level occur in species for which the group level is potentially ephemeral and easily broken-up? Would not the existence in the single-cell state select for traits that are optimal at the single-cell level? Much remains to be explored with respect to the connections between divisibility and adaptedness of a higher level as it first emerges.

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Tables

Table 1: Description of symbols used

Symbol	Description	Dimensions
a	Parameter determining how much effect the colony level of organization has on the growth trajectory of a cell. $0 \leq a \leq 1$.	-
B	Average resting metabolic rate of an organism.	mass length^2 time^{-3}
B_0	A taxon-specific constant related to the scaling of metabolic rate on mass.	mass$^{(1-\gamma)}$ length^2 time^{-3}
c_1	Constant used to relate light level to cell growth rate.	length^2 amount^{-1}
c_2	Constant used to relate light level to cell growth rate.	time^{-1}
c_3	Constant used to relate light level to cell asymptotic size.	volume
c_4	Constant used to relate light level to cell asymptotic size.	length^2 time amount^{-1}
CP	Commitment point. The point in the cell cycle after which a cell divides even in the absence of light.	-
f	Parameter that controls the fold-growth needed at t_{chk} for a cell to have a particular target size.	-
G_0	Gap 0, a phase of suspended activity that cells enter when conditions are not conducive to growth.	-
G_1	Gap 1, the phase during which cells grow.	-
G_2	Gap 2, the phase between synthesis and mitosis. Assumed to be negligible here.	-
k	Given by $\frac{c_1 Q + c_2}{\ln(2) + (c_1 Q + c_2) t_{\text{div}}}$.	time^{-1}
m	Mass of an organism.	mass
n	Number of rounds of cell division per G_1 phase. Because all cell lineages undergo the same number of divisions, the number of offspring cells per cell per G_1 phase is 2^n.	-
$\bar{n}$	Average number of rounds of cell division per G_1 phase over a population of variable cells.	-
Q	Light intensity. Photons of photosynthetically active radiation per area per time.	amount length^{-2} time^{-1}
r	Exponential volume growth rate of cell.	time^{-1}

r_{pop}	Exponential growth rate of a population of clones. Used as a proxy for fitness.	time^{-1}
S/M	Refers to one round of DNA synthesis (S) and mitosis (M).	-
t	Time.	time
T	The total interdivision time. The time from the end of one round of divisions to the end of the next round of divisions.	time
t_{chk}	The time, measured from the start of growth, at which a cell checks its size. The size at this time is used to determine the target size of the cell.	time
t_{dly}	Length of the time delay between attainment of commitment and initiation of division.	time
t_{div}	The time that one round of cell division takes. Growth does not occur during this time.	time
t_{grw}	The duration of the uninterrupted growth phase of the cell.	time
t_{oth}	A fixed amount of time that occurs once per generation. Growth and division both do not occur during this time.	time
V	Volume of a cell.	volume
V_0	Initial volume of cell (i.e., volume at hatching).	volume
V^{C1}	Volume of an in-name-only colony. These colonies are recognizable groups of cells, but each cell grows exactly as it were a unicell.	volume
V_1	Volume of a cell within an in-name-only colony.	volume
V^{C2}	Volume of a giant-cell-like colony. These colonies have recognizable subdivisions (cells), but they grow the same as a giant cell (with no subdivisions) would.	volume
V_2	Volume of a cell within a giant-cell-like colony.	volume
V_{f}	Final volume of a parent cell. Volume of the cell after the growth phase and just before initiation of divisions.	volume
V_{g}	Volume of a cell within a “general” colony, with growth behavior intermediate to the in-name-only colony and giant-cell-like colony.	volume
V_{min}	Smallest possible volume of an offspring cell.	volume
V_{tar}	The target size of a cell. Given by $V_0 2^n$, where n is an integer.	volume

	Parameter in the logistic cell growth trajectory.	
V_∞	Asymptotic cell volume as $t \to \infty$. This parameter is assumed to increase exponentially with increasing light levels.	volume
x	Number of cells in a population.	cells
x_0	Initial number of cells in a population.	cells
α	The base 2 logarithmic growth rate of a population of cells .	time^{-1}
δ	The doubling rate of a population of cells.	time^{-1}
θ	rt_{chk}.	-
μ	Mean of rt_{chk} in a population of variable cells.	-
ρ	Parameter in the logistic cell growth trajectory. If $V_\infty \to \infty$, cell growth is exponential and ρ is the exponential growth rate. This parameter is assumed to increase linearly with increasing light levels.	time^{-1}
σ^2	Variance of rt_{chk} in a population of variable cells.	-
Ψ	The probability density function of rt_{chk}.	

Table 2: Comparison of reaction norm of optimal n as a function of cell growth rate for different costs and benefits of changing n.

Cost of higher n (i.e. benefit of lower n)	Cost of lower n (i.e. benefit of higher n)	Hierarchical level of size-dependent growth	Formula for fitness, r_{pop}	Optimal reaction norm of n on cell growth rate
None	None	n/a	$\left(\frac{1}{r}+\frac{t_{\text{div}}}{\ln(2)}\right)^{-1}$	Fitness is independent of n at all cell growth rates/light intensities.
None	Switching time (t_{oth})	n/a	$\left(\frac{1}{r}+\frac{t_{\text{div}}}{\ln(2)}+\frac{t_{\text{oth}}}{n\ln(2)}\right)^{-1}$	Fitness is always maximised at highest possible n.
Growth trajectory in which growth rate is a decreasing function of size	None	Cell	$\left(\frac{1}{\rho}+\frac{1}{n\,\rho\,\ln(2)}\ln\left(\frac{V_0-V_\infty}{2^nV_0-V_\infty}\right)+\frac{t_{\text{div}}}{\ln(2)}\right)^{-1}$	Fitness is always maximised at lowest possible n.
Growth trajectory in which growth rate is a decreasing function of size	Switching time (t_{oth})	Cell	$\left(\frac{1}{\rho}+\frac{1}{n\,\rho\,\ln(2)}\ln\left(\frac{V_0-V_\infty}{2^nV_0-V_\infty}\right)+\frac{t_{\text{div}}}{\ln(2)}+\frac{t_{\text{oth}}}{n\ln(2)}\right)^{-1}$	Optimal reaction norm varies with switching cost (t_{oth}). See Figure 11.
Growth trajectory in which growth rate is a decreasing function of size	Switching time (t_{oth})	Colony (group)	$\left(\frac{1}{\rho}+\frac{1}{n\,\rho\,\ln(2)}\ln\left(\frac{V_0-aV_0+2^naV_0-V_\infty}{2^nV_0-a2^nV_0+2^{2n}aV_0-V_\infty}\right)+\frac{t_{\text{div}}}{\ln(2)}+\frac{t_{\text{oth}}}{n\ln(2)}\right)^{-1}$	Optimal reaction norm varies with switching cost (t_{oth}) and level of group effect on cell growth (a). See Figure 11.

Figure Legends

Figure 1. Family-level phylogeny of colonial volvocine algae. Unicellular *C. reinhardtii* is represented as an out-group. Families are as described in Nozaki (2003). Range of species-level maximum n is shown for each family. Maximum n values are from Coleman (2012) and references therein. Micrographs show examples of body forms. From top to bottom, *Volvox carteri*, *Gonium pectorale*, *Basichlamys sacculifera*, and *Chlamydomonas reinhardtii* are shown. Scale bars are 10 μm in the bottom three images and 50 μm in the top image.

Figure 2. A) A multiple fission/palintomy asexual cell cycle is depicted. The number of rounds of synthesis and mitosis (S/M) that occur in one cycle is n. G_1 is the prolonged growth phase, during which cells can grow from 2-fold to more than 30-fold in volume. G_0 is a resting phase that occurs after divisions if conditions are not conducive to cell growth. CP is the commitment point, defined as the point after which cells exposed to prolonged darkness undergo at least one round of divisions. The volume of the cell, which ranges from V_0 to $2^n V_0$, is indicated. B) In undifferentiated colonial volvocine algae, the asexual cell cycle is fundamentally similar to the *Chlamydomonas* cycle. Note that from the perspective of one cell within a colony, the cell cycle is essentially the same as in the unicellular case. There are at least two major differences between the two cases. First, the microenvironment surrounding a focal cell is different in the colonial case because each cell is in the immediate vicinity of the other offspring cells that resulted from its parent's divisions. Thus, in addition to cell volume (which varies similarly as in the unicellular case), cells within a colony also may experience effects based on total colony size, which varies from $2^n V_0$ to $2^{2n} V_0$. Second, in the colonial case, some mechanism for cells or offspring colonies to separate is in place and keeps the colony from continually increasing in size

as cell generations pass. This is not necessarily the same mechanism that allows offspring cells to separate in the unicellular case.

Figure 3. The reaction norm of n on light intensity is plotted along with the reaction norm of $\log_2$(final size) on cell exponential growth rate. The two y-axes show the assumption that $V_\mathrm{f} = V_0 2^n$. The n values are as reported in Vítová et al. (2011b), their Table 1 (see Appendix 1). The corresponding V_f values were calculated with the assumption that $V_0 = 45$. The light intensities of populations with varying n-values were directly reported in Vítová et al. (2011b), and the black data points show this information. For each of these populations, we estimated the cell exponential growth rate (as described in Appendix 1). The data for cell exponential growth rate versus final size/n is shown in gray. Cell exponential growth rate and light intensity were linearly related, but the relationship was not perfect ($R^2 = 0.95$). The error in the linear relationship between light intensity and cell growth rate creates some horizontal distance between points representing the same population (shown by nearby black and gray pairs with the same type of symbol). The gray solid and black dashed line show the approximately linear relationship between cell growth rate and $\log_2$(final size)/n and between light intensity and $\log_2$(final size)/n, respectively. The reaction norm of n on light intensity is our relationship of interest because changes in this relationship would constitute changes in the regulation of cell number. This relationship is approximately equal to the relationship between cell growth rate and $\log_2$(final size). The latter kind of relationship is relevant to understanding how a cell might regulate n through sizers and timers (see Figure 4).

Figure 4. A) If an absolute sizer is the cause of the second of two events (e.g. cell division), then the size at event two (final size) should be constant across different growth rates. The slope of the time between events (duration of growth) as a function of growth rate should be -1 on a log-

log scale. The final size should also be constant across varying durations of growth. In the example shown, $V_f = 90$. That is, cells grow exponentially until they attain a volume of 90 μm^3 and they initiate divisions in response to attainment of this volume. B) If an absolute timer is the cause of the second of two events, then as growth rate increases, the logarithm of the size at event two (final size) should increase with a slope equal to the length of the absolute timer. As growth rate increases, time between events (duration of growth) should remain constant. There should be no (or very little) variation in duration of growth, even when size at event two varies substantially (due to different growth rates). In this example, the length of the timer is 12. That is, cells divide after 12 h of growth for all growth rates. All of these relationships follow from assuming that the size at event two is the result of exponential growth during the period from event one to event two. C) The pattern in *C. reinhardtii* rules out an absolute timer alone or an absolute sizer alone because neither size at divisions nor time of divisions is constant across differing growth rates. Note that although the logarithm of final size increases approximately linearly with growth rate, the slope of this relationship cannot be interpreted as the duration of a timer (as in the second column) because the duration of growth is not constant with growth rate. The bottom panel shows that cell division regulation approximates an absolute timer pattern when growth rate is high (low duration of growth) and approximates an absolute sizer pattern when growth rate is low (high duration of growth). Note that once any two of growth rate, final size, and duration of growth are specified, the third can be calculated (assuming exponential cell growth). Data from all panels are interpreted from Vítová et. al (2011b) as described in Appendix 1. In the bottom two panels, diamonds (and a dotted line) indicate data points in which the cell cycle duration reported in Vítová et. al (2011b) is assumed to be the duration of growth. This is likely a slight overestimate of duration of growth. The circles (and a dashed line)

represent a growing time that was calculated from the reported n value and the inferred cell growth rate value. Final size was calculated from n using $V_0 = 45$.

Figure 5. A) The standard model of cell behavior in constant light is that the duration of growth (and final size/n) is determined by a sizer followed by a timer. The sizer is the period from the initiation of growth to the commitment point, which occurs at two-fold growth. The timer is a period of 5−8 h between the commitment point and initiation of divisions. B) The proposed model supposes that there is a constant (growth rate-independent time) during which division will not occur. This is the first timer part of the cycle and the end of this period occurs at t_{chk}. Based on the cell size at t_{chk}, a target size is established and growth continues until the target size is attained. Thus, the period between t_{chk} and the initiation of divisions is best described as a sizer, though it is important to note that the period between the start of growth and divisions is not governed by an absolute sizer. The commitment point could occur before or after t_{chk}. Its timing is not relevant to regulation of duration of growth in constant light according to the proposed model. C) The standard model applied to cells in a light/dark cycle implies that (at least sometimes), the internal cell mechanisms (the sizer and timer) do not determine the duration of growth. If growth occurs during the daylight, then the length of the day is the only determinant of the duration of growth. Duration of growth affects final size/n so external control of duration of growth is external control of n.

Figure 6. A) Relationships between growth rate, final size, and duration of growth are shown for the standard model with $t_{\mathrm{dly}} = 9$ and $V_0 = 35$. In the standard model, there is not a large difference between the behavior of the cell and the behavior of the population so we do not distinguish the two here. B) The single-cell level expectations for the proposed model are shown for $V_0 = 45$, $t_{\mathrm{chk}} = 6$, and $f = 0.5$. C) The population level expectations for the proposed model

are shown for $V_0 = 45$, $t_{\mathrm{chk}} = 6$, $f = 0.5$, and $CV = 0.3$. Throughout this figure, growth is assumed to be exponential and data points are from Vítová et al. (2011b) as described in Appendix 1.

Figure 7. A) The standard model predicts that at the single-cell level, final cell size varies independently of target sizes (sizes corresponding to integer-fold growth). The dotted lines indicate the sizer phase, during which cells grow to two-fold size. The double lines indicate the timer period, during which cells grow during a constant delay, measured from the attainment of two-fold growth. The inset shows that a unimodal distribution of final cell sizes is expected based on the standard model, given that cell growth rate and delay duration are random variables. Note that the inset distributions are not distributions of cell sizes at a particular time. They are distributions of cell sizes at a particular cell cycle benchmark (initiation of division), making them more difficult to assay at the population level. B) The GR-TS model is based on the idea that at the single-cell level, cells transition from growth to division at a target size. This model implies a multimodal distribution of cell size-at-division (inset). Matsumura et al. (2010) present data that is consistent with this pattern. C) The single-cell level check time model was used to calculate the post-commitment time as a function of cell growth rate (blue line). Periods of decline indicate that, over a range of growth rates that give rise to one particular n value, post-commitment time declines with growth rate. The least upper bound of post-commitment time is t_{chk}, which in this example is 10. The standard model suggests that the post-commitment time is constant with random deviations (i.e. no oscillations) (shown in red). Data from Vítová et al. (2011b) are also shown.

Figure 8. A-C) The results of the population-level check time model. Values of t_{chk} from 2 h to 12 h are indicated by colors (see legend in panel A). CV is the relative variation in size at $t =$

t_{chk}. D-F) These are the same as previous three panels except a new parameter, f was included in the model. This parameter affects the relationship between fold-growth at $t = t_{\text{chk}}$ and target volume/n value. Here $f = 0.5$.

Figure 9. The effect of the parameter f is shown. In both panels, the probability density function of rt_{chk} (i.e. $\Psi(\theta)$ is shown). In this example (both panels), average $rt_{\text{chk}} = 0.9$ and the standard deviation of rt_{chk} is 0.27 ($CV = 0.3$). The vertical lines show the cut-off values that relate variation in rt_{chk} to variation in n. For example, in the top panel (when $f = 0$), a cell must grow at least 2-fold but not more than 4-fold by $t = t_{\text{chk}}$ in order to divide for two rounds ($n = 2$). That is, $2 < e^{rt_{\text{chk}}} \leq 4$. Thus, the vertical lines delineating cells with an n value of 2 are at ln(2) and ln(4) in the upper panel. By contrast, in the lower panel, a cell must grow at least 1.2-fold but not more than 2.4-fold by $t = t_{\text{chk}}$ in order to have $n = 2$ (i.e. the vertical lines are at $\ln(2) - 0.5$ and $\ln(4) - 0.5$). More generally, $n = k$ when $\ln(2^{k-1}) - f < rt_{\text{chk}} \leq \ln(2^k) - f$.

Figure 10. In all panels, fitness values as a function of $\left(\frac{1}{T}\right)$ and n are indicated by colors, with red indicating highest fitness and blue indicating lowest fitness. Fitness is calculated as $\left(\frac{1}{T}\right) n \ln(2)$ and only fitness values up to 0.25 are represented. That is, fitness continues to increase in the upper right corner of the graph, though this is not shown. In all panels, white lines indicate the combinations of $\left(\frac{1}{T}\right)$ and n that are possible based on specific assumptions about cell growth and division. Different white lines correspond to different levels of light intensity (i.e. different levels of resource input). Gray points indicate the combination of $\left(\frac{1}{T}\right)$ and n that gives the highest fitness (reddest color) for a given set of constraints (light level, assumptions about growth). A) Fitness as a function of $\left(\frac{1}{T}\right)$ and n. Higher values of $\left(\frac{1}{T}\right)$ and of n lead to higher fitness. Gray lines are lines of equal fitness. B) White lines indicated the case of exponential cell

growth and zero switching time. The possible values of $\left(\frac{1}{T}\right)$ and n that a cell can attain for a given resource input follow the lines of equal fitness. ($t_{\text{div}} = 1$, $t_{\text{oth}} = 0$, $c_1 = 0.00095$, $c_2 = 0.02135$) Fitness depends only on light level, not on the cell's choice of n. Light levels (from lowest to highest white line) are 60, 110, 160, 210, and 260 μmol m^{-2} s^{-1}. C) White lines correspond to the case of exponential cell growth and non-zero switching time (same parameter values as in A, except $t_{\text{oth}} = 3$). In this case, cells attain the highest fitness by maximizing n. D) White lines correspond to the case of sigmoidal (logistic) cell growth and zero switching time. (The parameter values are the same as in A with the additional parameters $c_3 = 81.369$ and $c_4 = 0.021$) In this case, cells attain the highest fitness by minimizing n. E) White lines correspond to the case of sigmoidal (logistic) cell growth and non-zero switching time. (Same parameter values as in D, except $t_{\text{oth}} = 1$.) In this case, cells attain the highest fitness by having n values that increase with light intensity. That is, higher white lines have a higher optimal n. F) White lines correspond to the case of sigmoidal (logistic) cell growth and non-zero switching time and a detrimental effect of colony size on cell growth. (The parameter values are the same as in E with the additional parameter $a = 0.95$.) The optimal n at high light levels is lower in this case compared to the case in which colony size did not affect cell growth.

Figure 11. Optimal reaction norm of n on light intensity is affected by the cost of low n (t_{oth}). Presumably, the relationship between growth parameters (ρ and V_∞) and light levels (i.e. the benefit of low n) also affects the optimal reaction norm, though we did not investigate this here. The blue lines ($a = 0$) show optimal reaction norm when cell growth is slowed as a function of cell size. The red lines ($a > 0$) show the optimal reaction norm when cell growth is slowed as a function of colony size. Higher values of a correspond to a larger effect of the colony on cell growth.

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Figures

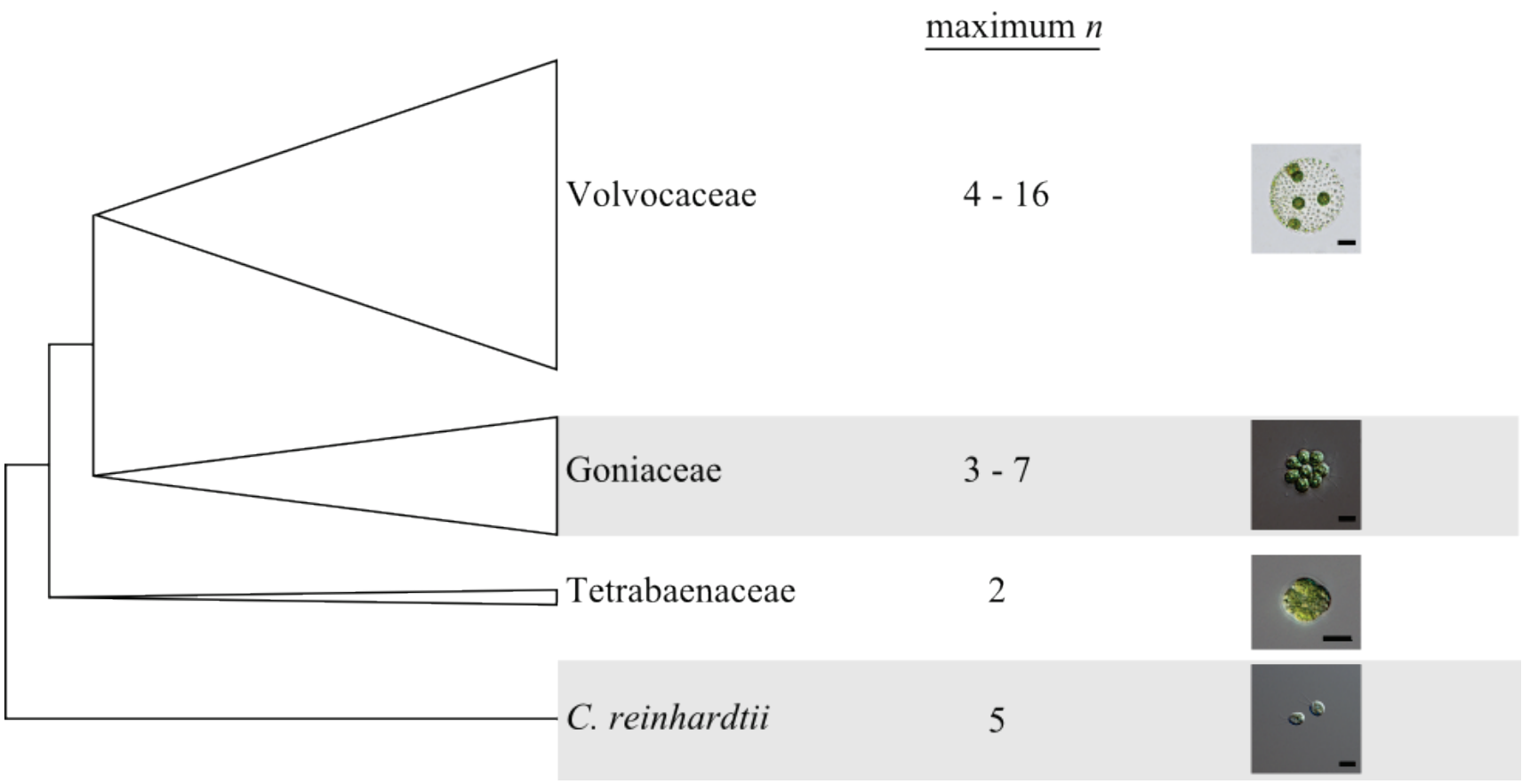


Figure 1

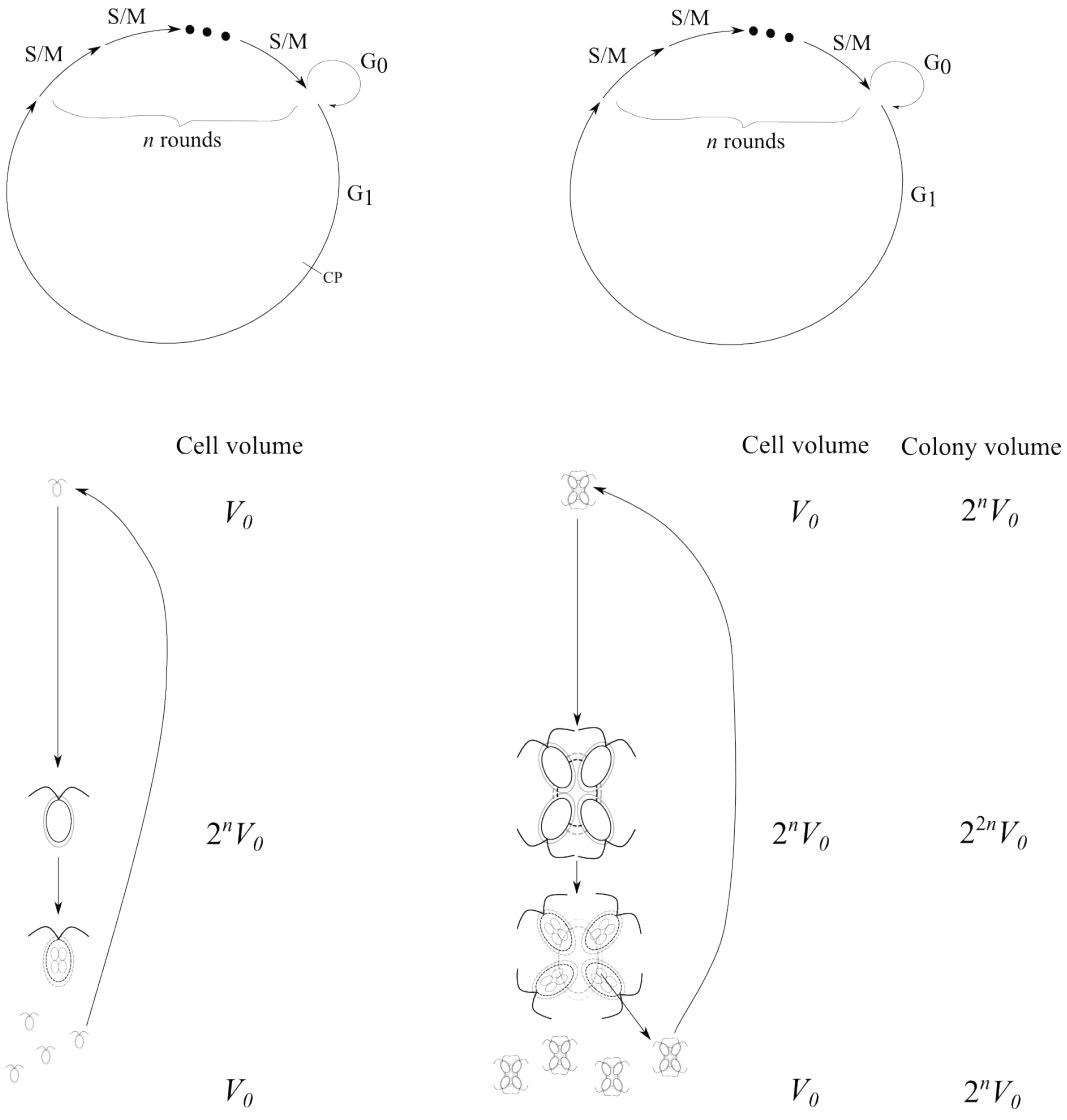

A) Palintomy
B) Autocolony
S/M
S/M
S/M
G0
n rounds
G1
CP
Cell volume
Colony volume
V_0
$2^n V_0$
$2^{2n} V_0$

Figure 2

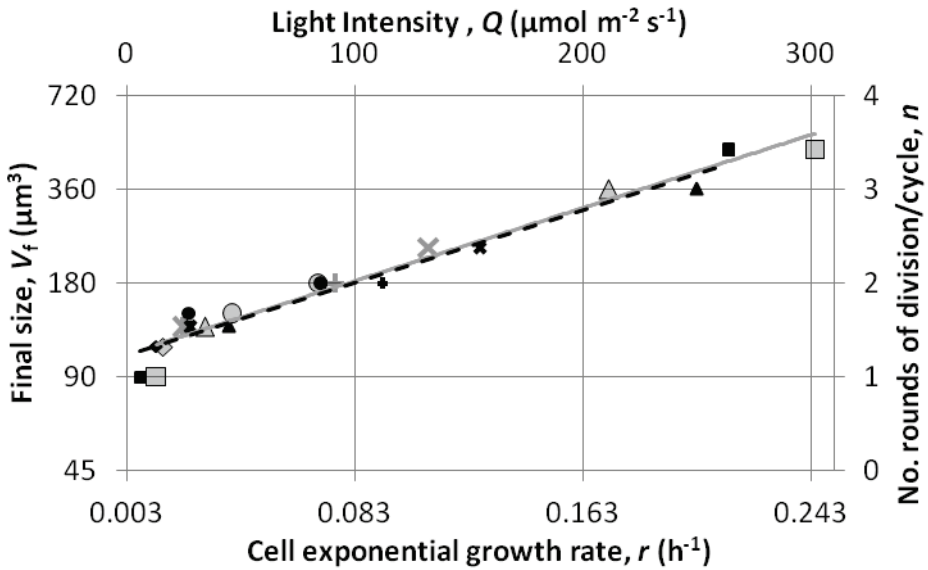

Light Intensity , Q (µmol m-2 s-1)
0
100
200
300
720
360
180
90
45
Final size, Vf (µm3)
4
3
2
1
0
No. rounds of division/cycle, n
0.003
0.083
0.163
0.243
Cell exponential growth rate, r (h-1)

Figure 3

A) Patterns indicative of an absolute sizer

B) Patterns indicative of an absolute timer

C) *C. reinhardtii* pattern (from data in Vítová et. al 2011, their Table 1)

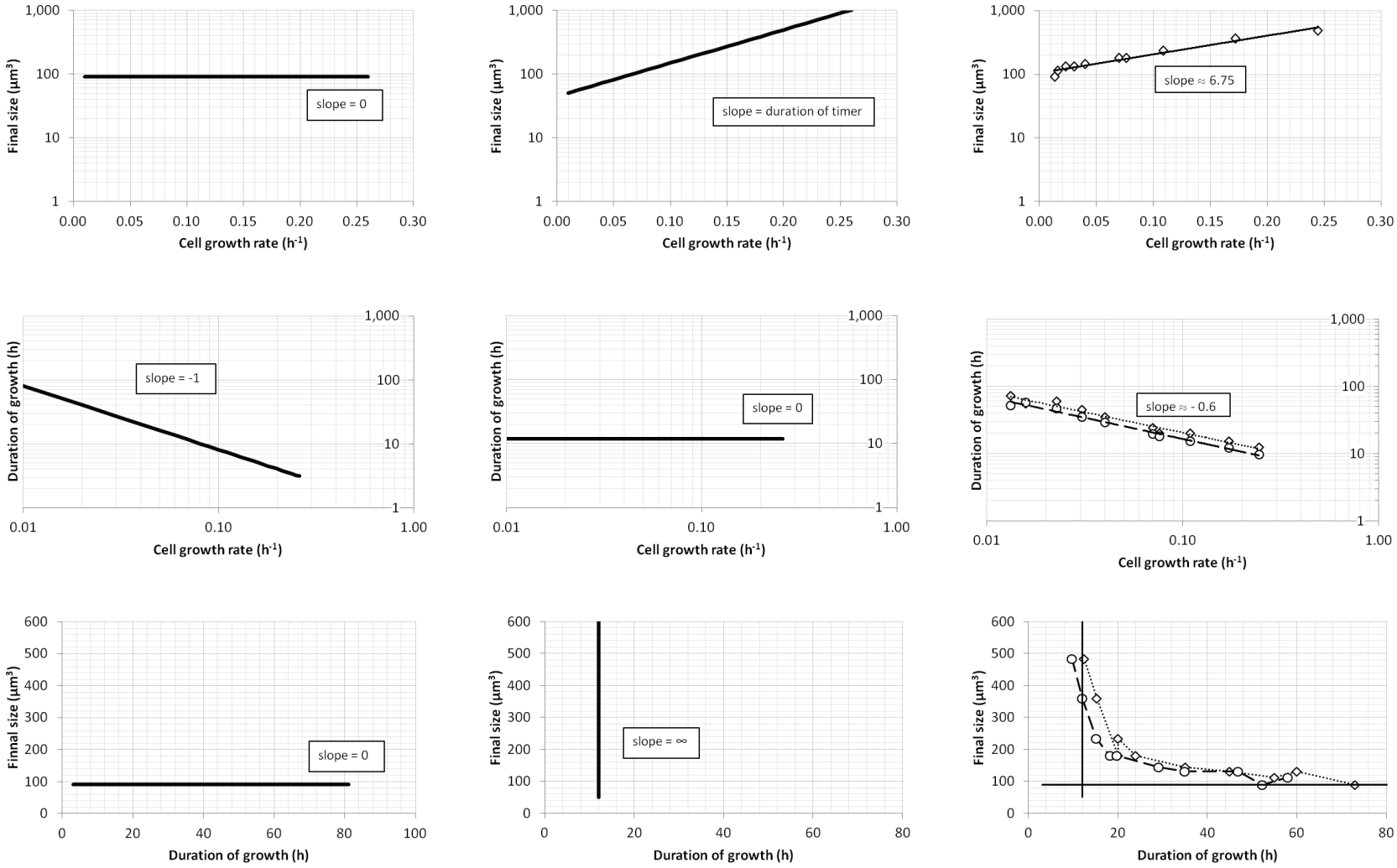


Figure 4

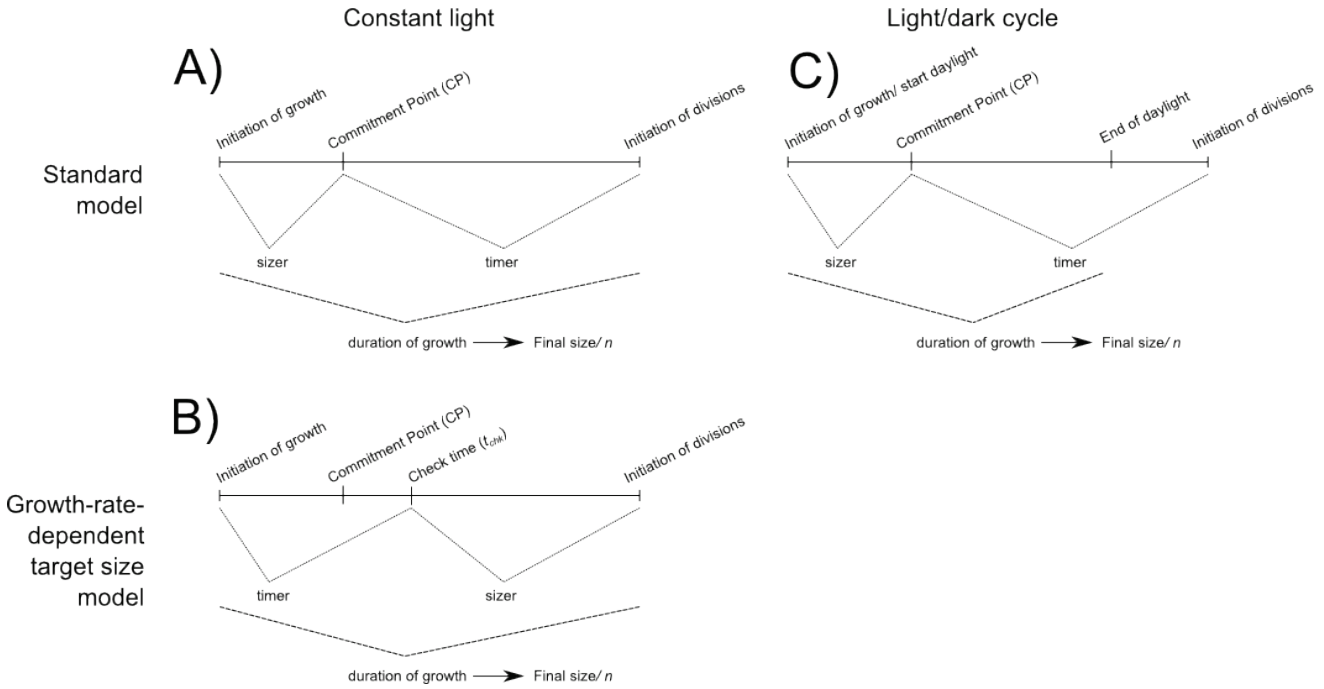

Constant light
Light/dark cycle
A)
C)
Standard model
Initiation of growth
Commitment Point (CP)
Initiation of divisions
sizer
timer
duration of growth
Final size/ n
Initiation of growth/ start daylight
End of daylight
B)
Growth-rate-dependent target size model
Check time (t_{chk})

Figure 5

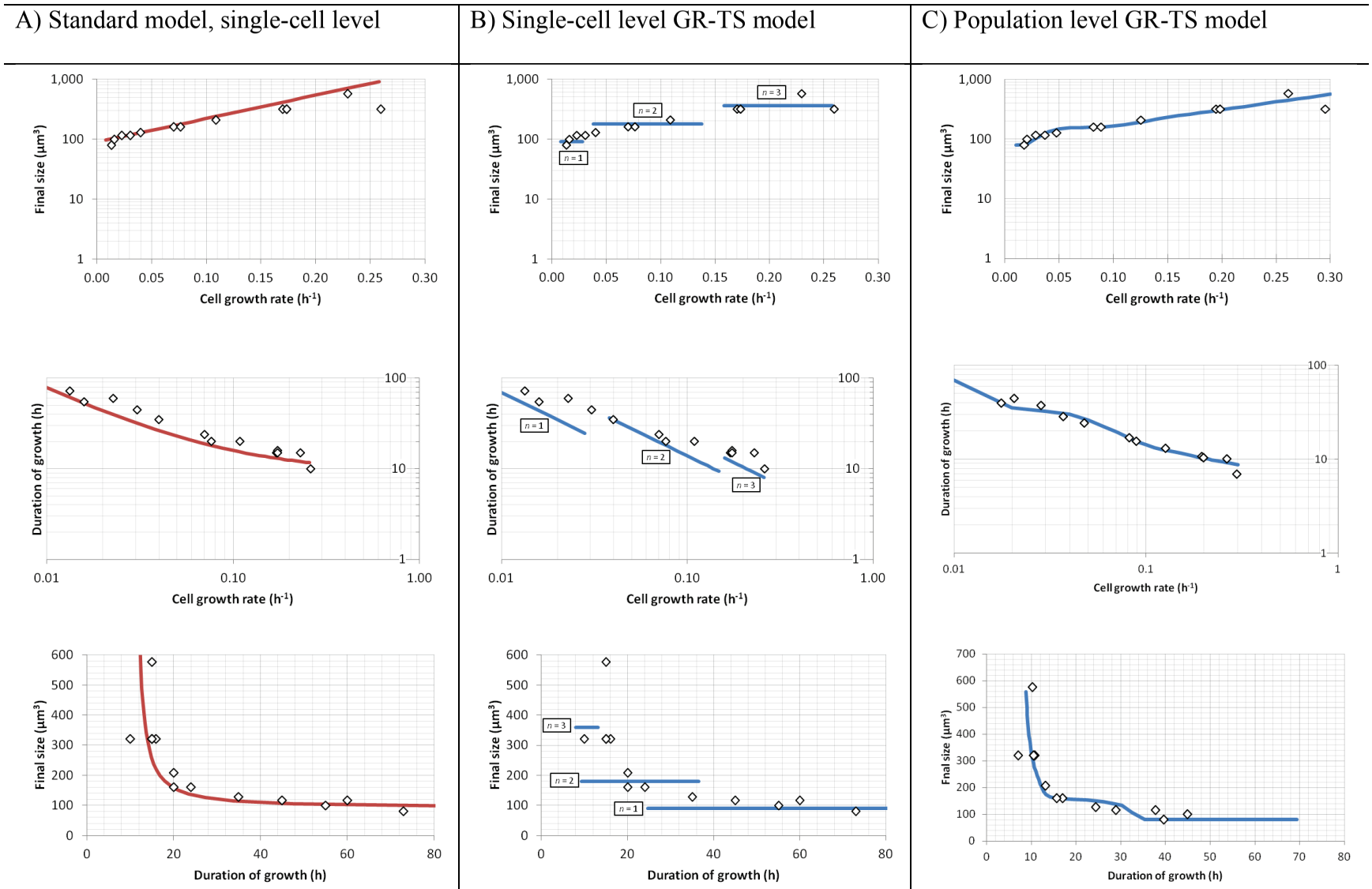
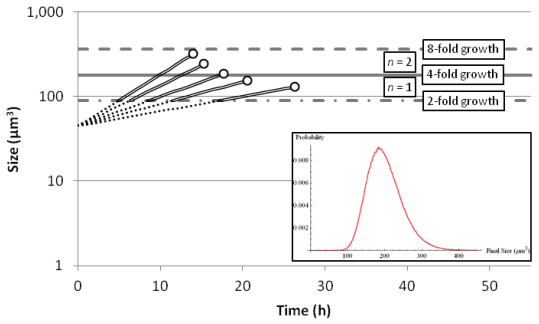
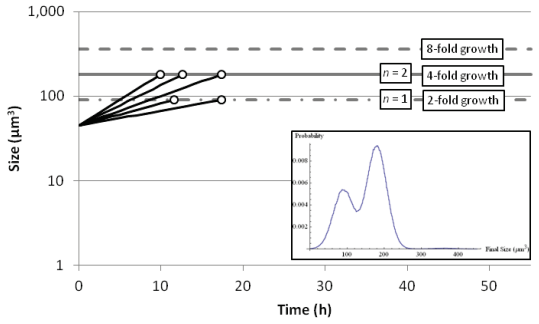

A) Standard model, single-cell level
B) Single-cell level GR-TS model
C) Population level GR-TS model
Final size (µm³)
Cell growth rate (h⁻¹)
Duration of growth (h)
Cell growth rate (h⁻¹)
Final size (µm³)
Duration of growth (h)
n = 1
n = 2
n = 3
Fnal size (µm³)

Figure 6

A)


1,000
100
10
1
Size (µm³)
0
10
20
30
40
50
Time (h)
8-fold growth
4-fold growth
2-fold growth
n = 2
n = 1

B)


1,000
100
10
1
Size (µm³)
0
10
20
30
40
50
Time (h)
8-fold growth
4-fold growth
2-fold growth
n = 2
n = 1

C)

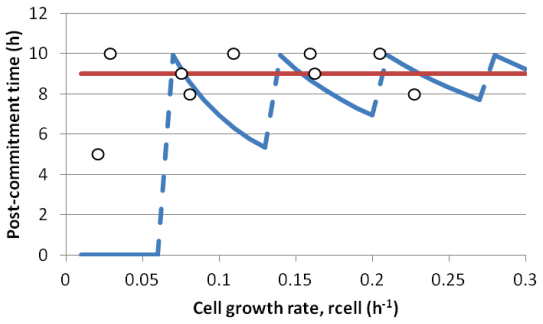

12
10
8
6
4
2
0
Post-commitment time (h)
0
0.05
0.1
0.15
0.2
0.25
0.3
Cell growth rate, rcell (h-1)

Figure 7

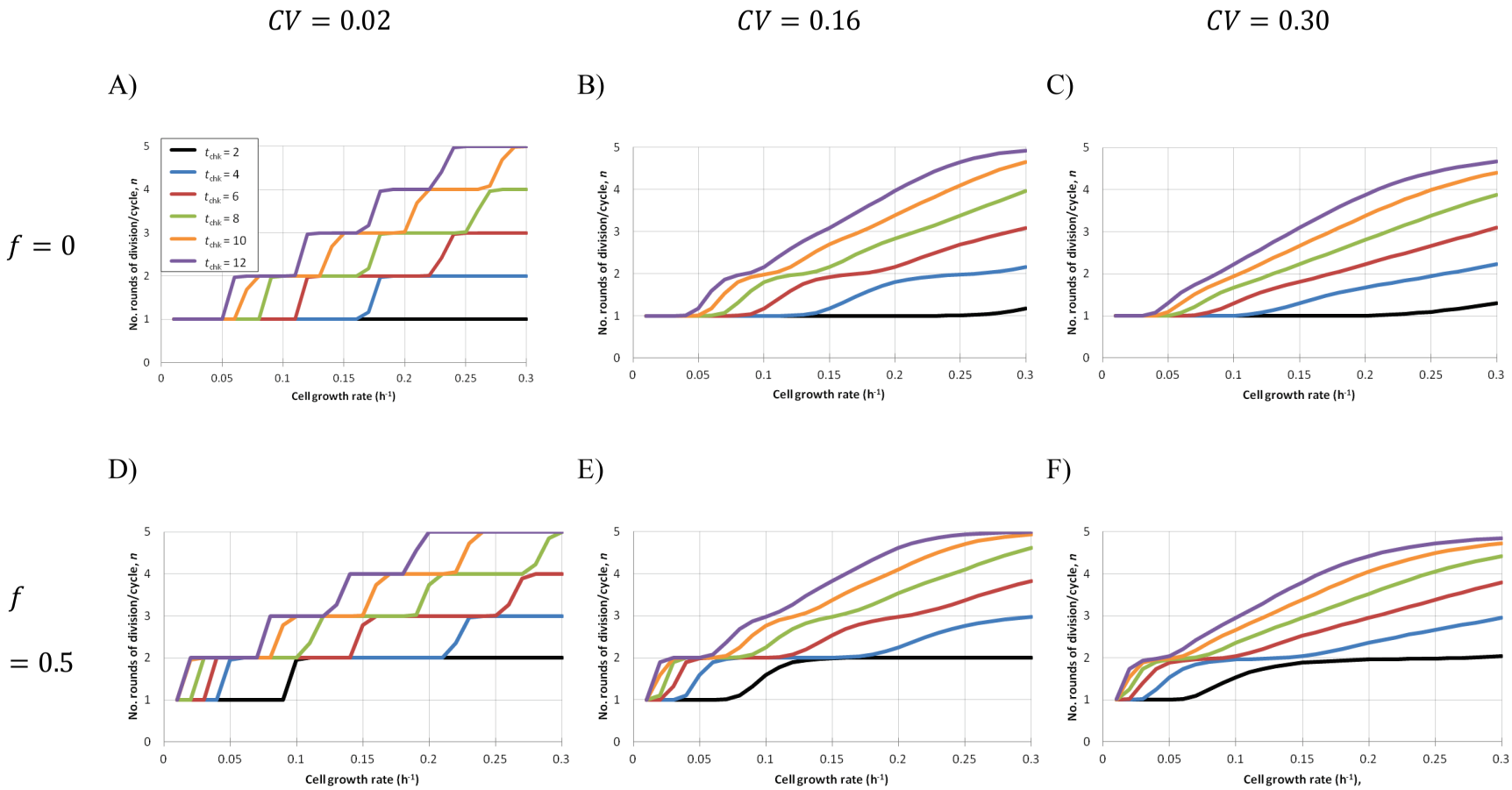

$CV = 0.02$
$CV = 0.16$
$CV = 0.30$
A)
B)
C)
D)
E)
F)
$f = 0$
$f = 0.5$
$t_{chk} = 2$
$t_{chk} = 4$
$t_{chk} = 6$
$t_{chk} = 8$
$t_{chk} = 10$
$t_{chk} = 12$
No. rounds of division/cycle, n
Cell growth rate (h-1)
Cell growth rate (h-1),

Figure 8

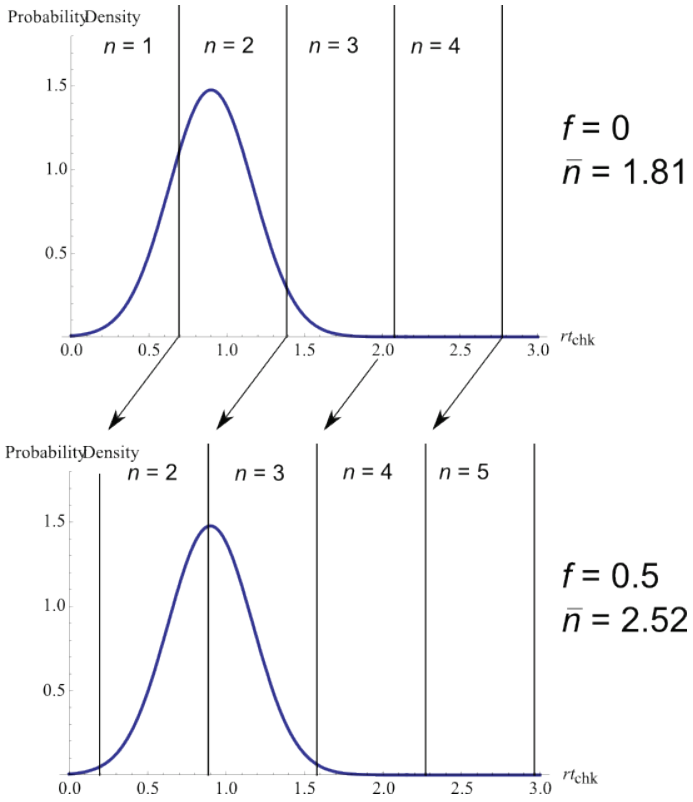

Probability Density
n = 1
n = 2
n = 3
n = 4
1.5
1.0
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
rt_chk
f = 0
n̄ = 1.81
Probability Density
n = 2
n = 3
n = 4
n = 5
1.5
1.0
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
rt_chk
f = 0.5
n̄ = 2.52

Figure 9

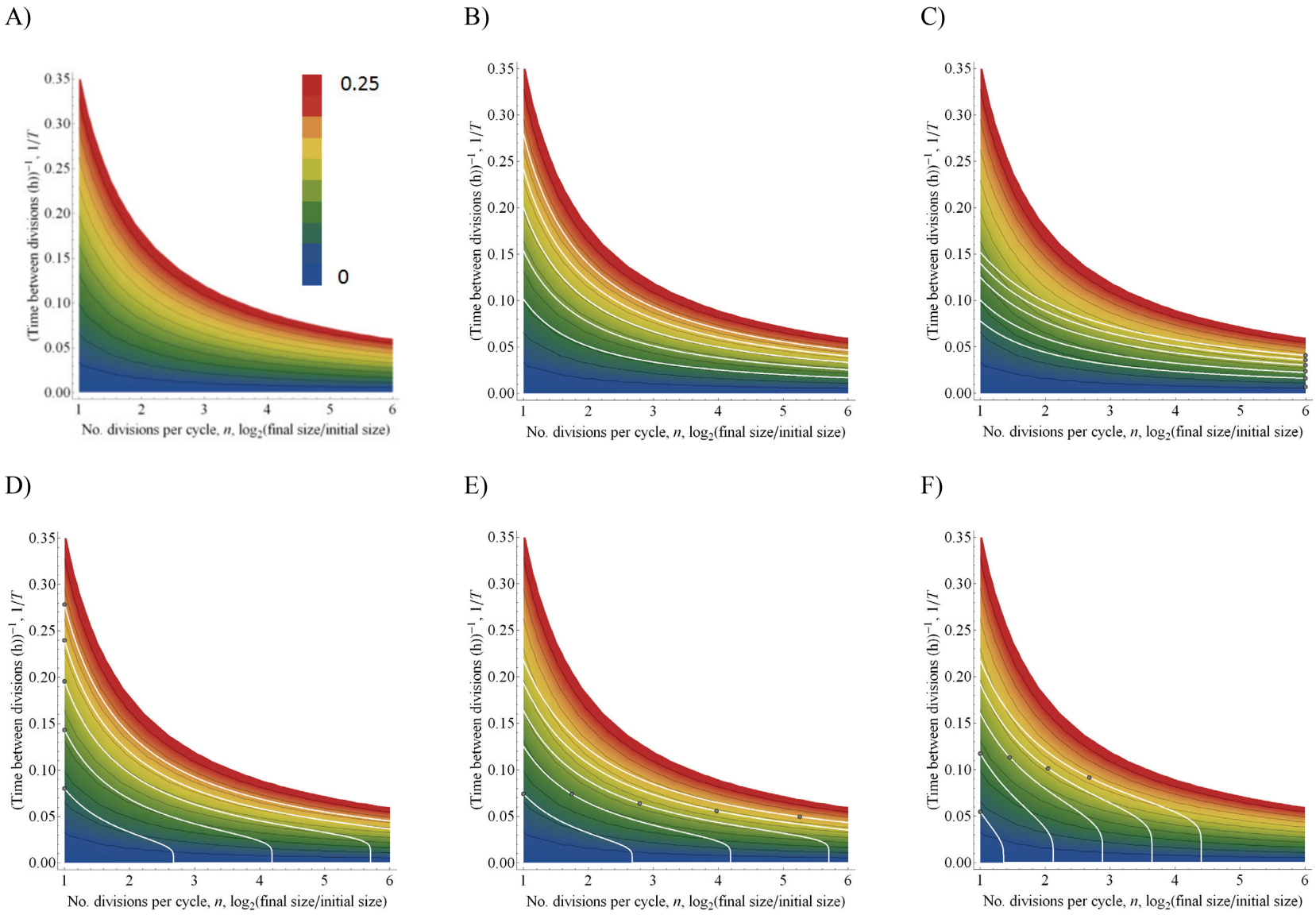

A)
B)
C)
D)
E)
F)
0.25
0
(Time between divisions (h))$^{-1}$, 1/T
No. divisions per cycle, n, log$_2$(final size/initial size)

Figure 10

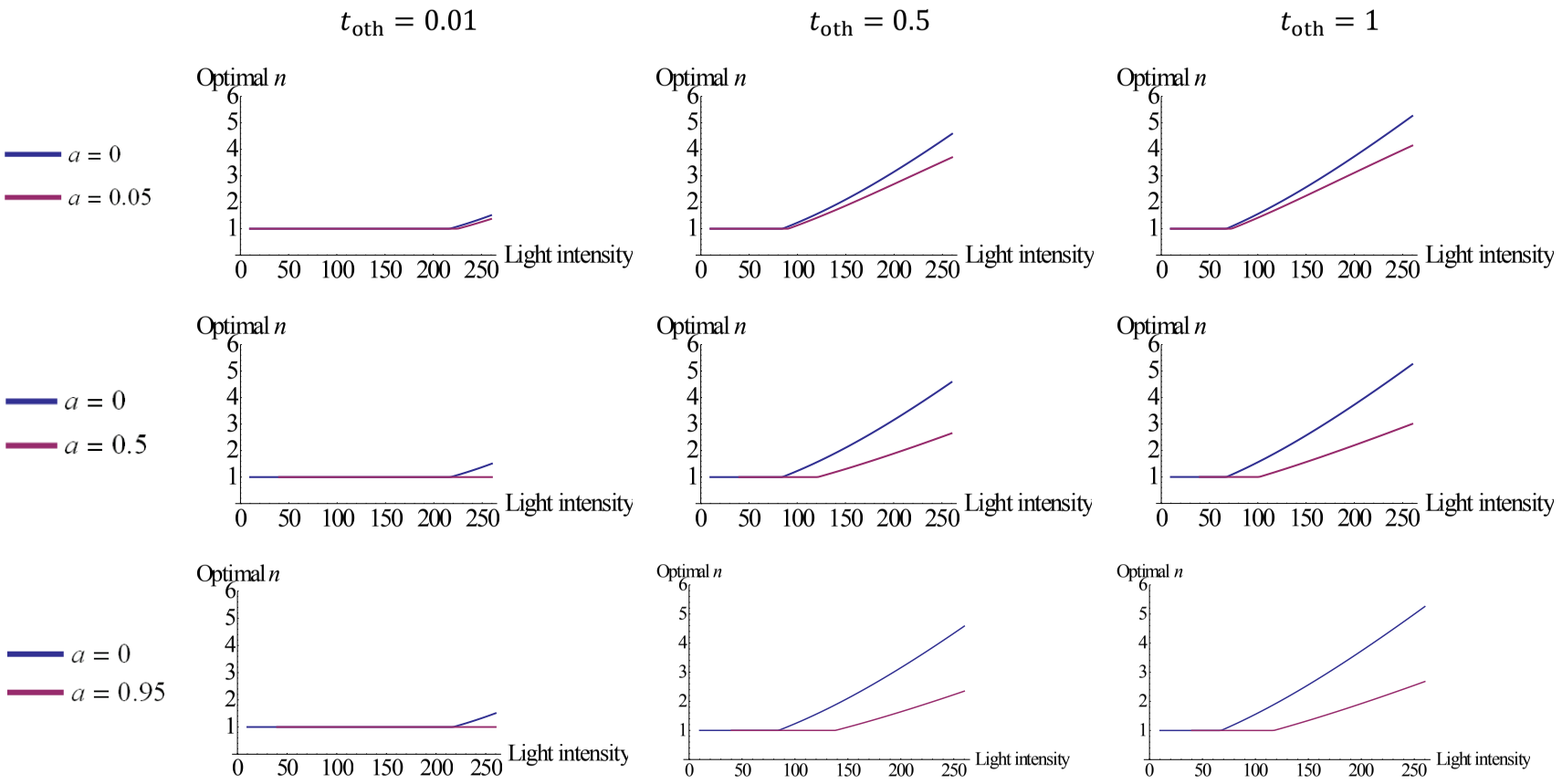

$t_{oth} = 0.01$
$t_{oth} = 0.5$
$t_{oth} = 1$
$a = 0$
$a = 0.05$
$a = 0$
$a = 0.5$
$a = 0$
$a = 0.95$
Optimal n
Light intensity
0 50 100 150 200 250
1 2 3 4 5 6

Figure 11

Appendix 1: Use of information from Vítová et al. (2011b)

We are interested in the reaction norm of n on light intensity (one example of a relevant environmental parameter) because regulation of this relationship amounts to regulation of "cell number". Values that make up this reaction norm are reported in Vítová et al. (2011b), their Table 1. We used the base 2 logarithm of daughter cell number to calculate n and plotted these values here in Figure 3. The relationship between n and light intensity is approximately linear. The best fit line is $n = 0.0077Q + 1.22$, where Q is light intensity in μmol m^{-2} s^{-1}, and $R^2 = 0.96$ (black dashed line, Figure 3). Several data points reported in Vítová et al.'s (2011b) Table 1 were excluded here. These include the points that were based on asynchronous cultures, data from the mutant *per1*, and data from cultures that were exposed to a period of dark during the pre-commitment phase. Average n was used for light intensities that were repeated for more than one population of cells. See Table A1 below for the reduced set of data from Vítová et al.'s (2011b) Table 1.

If cell exponential growth rate is linearly related to light intensity, then a linear relationship between light intensity and n implies a linear relationship between cell growth rate and the logarithm of final volume (Figure 3). The latter relationship is important because of its relevance to recognizing the effects of sizers and timers (Figure 4). We estimated cell exponential growth rate by fitting exponential growth curves to the growth trajectories depicted in Figure 3 of Vítová et al. (2011b). We used Engauge Digitizer (http://digitizer.sourceforge.net/) to estimate the data points shown in Vítová et al.'s (2011) Figure 3. The results of the exponential curve fitting are summarized in Table A2 below. Our direct estimates of cell exponential growth rate (r) at different light intensities from Vítová et al.'s (2011b) Figure 3 were linearly related to Vítová et al.'s (2011b) estimates of population growth rates at the same

light intensities (from their Table 1). The most basic expectation would be that $r = \ln(2)\,\delta$ where δ is the doublings per hour reported in Vítová et al. (2011b) (designated μ in Vítová et al. (2011b))[4]. However, the relationship between cell growth, cell reproduction, and population growth is not so simple if variation among cells is substantial (Painter and Marr 1967). Therefore, we estimated r directly from Vítová et al.'s (2011b) Figure 3 rather than calculating it from the reported δ. With our direct estimates of r, we found a best fit line of $r = 0.87\delta - 0.0012$ and $R^2 = 0.998$. This is somewhat close to the theoretical expectation if there were no variation within the population (i.e. slope of 0.69 and intercept of 0). For all populations, we used a linear relationship with a slope of 0.87 and an intercept of -0.0012 to estimate r from the δ value reported in Vítová et al.'s (2011b) Table 1. See Table A1. The exponential growth rates estimated in this way were related to light intensity by the following function: $r = 0.0008Q + 0.0031$ ($R^2 = 0.9485$).

Table A1 Reduced set of values used from Vítová et al.'s (2011b) Table 1. The values of n and r were calculated as described in the text.

From Vítová et al.'s (2011b) Table 1			From our calculations	
Light intensity (μmol m^{-2} s^{-1})	Number of offspring cells	Avg. population doubling rate, δ (h^{-1})	n	Avg. exponential cell growth rate, r (h^{-1})
6	2	0.014	1	0.013
13	2.5	0.017	1.3	0.016
27	3.2	0.045	1.7	0.040
28	2.9	0.025	1.5	0.023
45	2.9	0.034	1.5	0.030
85	4.0	0.080	2.0	0.070
112	4.0	0.087	2.0	0.076
155	5.2	0.125	2.4	0.109
250	8.0	0.199	3.0	0.172
264	11.2	0.283	3.4	0.244

[4] To see this, note that $2^n = e^{rT}$. Combine this information with the doubling rate of the population, $\delta = \frac{n}{T}$, from Eqn. 15 and 16 to get the expectation that $r = \delta \ln(2)$.

Table A2 Results of fitting exponential growth curves to data reported in Vítová et al.'s (2011b) Figure 3. We found a linear relationship between population doubling rate and cell exponential growth rate and used this relationship to estimate cell exponential growth rate for other populations.

From Vítová et al.'s (2011b) Table 1		From our analysis of Vítová et al.'s (2011b) Figure 3			
Light intensity (μmol m^{-2} s^{-1})	Avg. population growth rate, δ (h^{-1})	Average exponential cell growth rate, r (h^{-1})	Average starting volume, V_0 (μm^3)	Average R^2	Number of growth curves analyzed
13	0.017	0.018	45.0	0.946	2
27	0.045	0.033	40.7	0.937	4
112	0.087	0.081	58.5	0.950	4
264	0.283	0.244	40.9	0.990	5

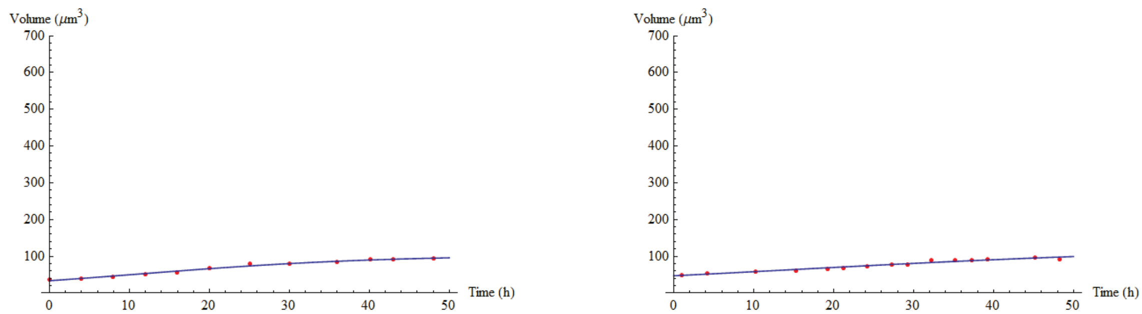
We were also interested in the effect of light intensity on cell growth trajectory if we made the assumption that the growth trajectories were sigmoidal rather than exponential. To explore this topic, we used the Mathematica function FindFit to fit a standard logistic curve to data from Vítová et al.'s (2011b) Figure 3. This iterative function did not usually converge, but the resulting parameter estimates are feasible nonetheless (see Figure A1). Our purpose here is not to find the growth function that best fits the data from Vítová et al. (2011b). Rather, our purpose is simply to uncover some realistic relationships between light level and logistic growth parameter values to include in our study of optimal n reaction norms. The logistic growth parameter estimates are in Table A3 and the fitted curves are shown in Figure A1. The maximum possible n for each growth curve was calculated as $\log_2\left(\frac{V_\infty}{V_0}\right)$.

Table A3 Results of fitting a standard logistic curve to growth data shown in Vítová et al.'s (2011b) Figure 3.

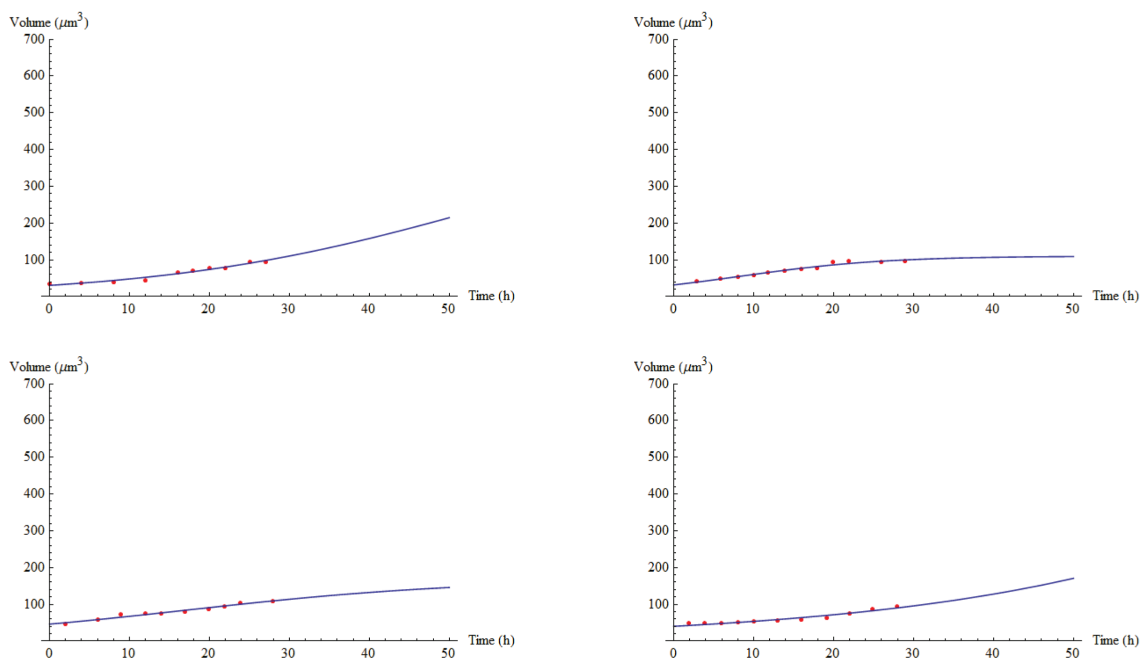
Light intensity (μmol m^{-2} s^{-1})	V_0 (μm^3)	V_∞ (μm^3)	ρ(h^{-1})	Max. possible n
13	33.5	103	0.07	1.6
	46.9	126	0.04	1.4
27	30.3	488	0.05	4.0
	31.6	110	0.11	1.8
	45.9	168	0.06	1.9

	40.1	1.2×10^7	0.03	18.2
112	47.4	307	0.14	2.7
	49.0	274	0.17	2.5
	64.7	430	0.09	2.7
	47.0	182	0.24	2.0
250	42.8	766	0.29	4.2
	46.1	3.6×10^7	0.23	19.6
	42.6	67,697	0.24	10.6
	35.8	3.2×10^7	0.24	19.8
	30.0	917	0.40	4.9

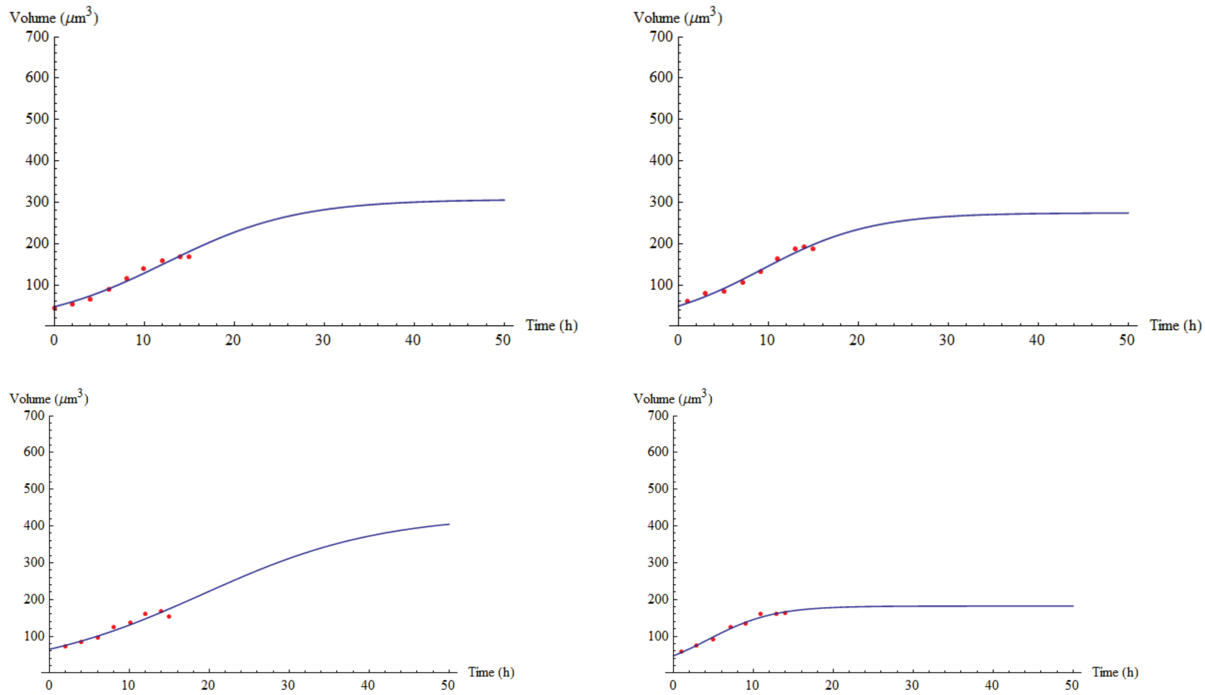
Light intensity = 13 μmol m^{-2} s^{-1}



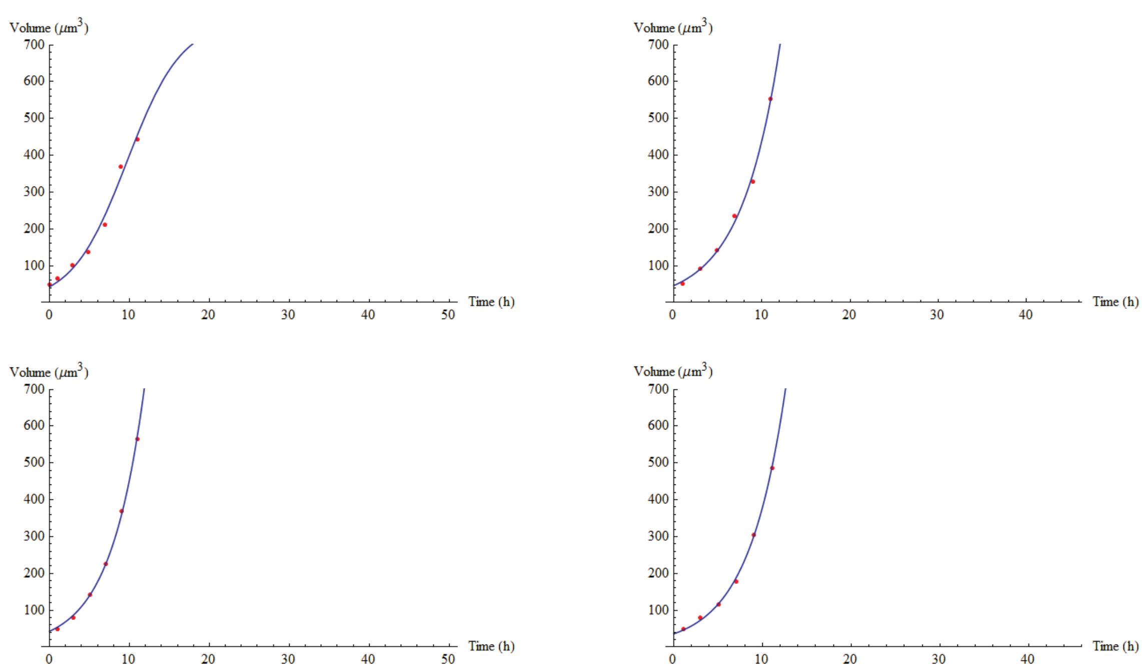
Light intensity = 27 μmol m^{-2} s^{-1}



Light intensity = 112 μmol m^{-2} s^{-1}



Light intensity = 250 μmol m^{-2} s^{-1}



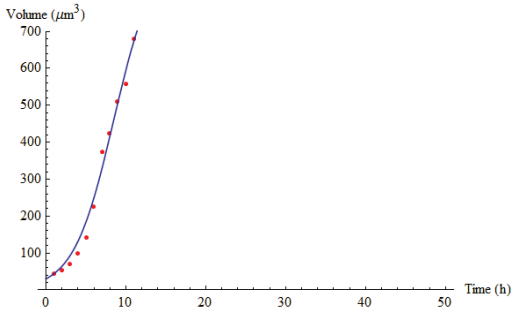


Figure A1 Logistic curves fit to growth data from Vítová et al.'s (2011b) Figure 3.

We used the logistic fitted functions to obtain reasonable relationships between the logistic growth parameters (ρ, V_∞) and light intensity (Q). We excluded growth curves for which the estimated V_∞ was $\sim 10^7$ and used the average parameter values for each light level. We then derived the following relationships: $\rho = 0.0011Q + 0.04$ $(R^2 = 0.9995)$ and $V_\infty = 81.4e^{0.021Q}$ $(R^2 = 0.907)$. In the main text, both r and ρ were related to light intensity by averaging their slopes and intercepts. That is, we used $r = \rho = 0.00095Q + 0.02155$ in order to make these two growth parameters directly comparable. We also used $V_\infty = 81.4e^{0.021Q}$ in the main text. The logistic curves for a variety of light intensities are plotted below (Figure A2).

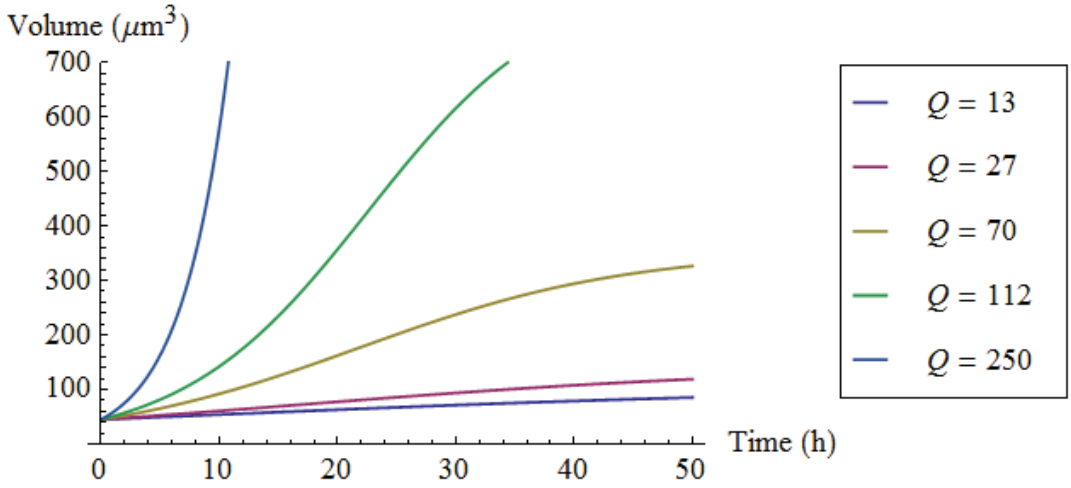


Figure A2 Logistic cell growth curves. Each curve shows cell growth at a different light intensity. Note that at $Q = 250$, V_∞ is very high ($\sim$15,500). Thus, at high light intensity, cell growth is close to exponential.

Appendix 2: Verbal models of asexual *Chlamydomonas* cell cycle regulation from the literature

Literature review methods

We conducted a Web of Science search for works that cite Craigie and Cavalier-Smith (1982). Craigie and Cavalier-Smith (1982) was chosen as the focal reference because this paper provides strong evidence for a key, uncontroversial aspect of *Chlamydomonas* cell cycle control (the dependence of the number of rounds of division on parental cell volume-at-division). Only the 19 results that were published within the last 10 years (2003 or later) were considered further. Of these, eight had a brief verbal summary of *Chlamydomonas* asexual cell cycle control (see below). Excluded from the below are seven works (Cavalier-Smith 2005; Holub et al. 2007; Lam et al. 2009; Matsumura et al. 2003a; Urzica et al. 2012; Valverde 2011; Yuan et al. 2013) that cite Craigie and Cavalier-Smith (1982) but have a minimal or no verbal summary of *Chlamydomonas* cell cycle control. Also excluded from the below are four articles (Matsumura et al. 2003b; Oldenhof et al. 2004; Oldenhof et al. 2006; Oldenhof et al. 2007) that cite Craigie and Cavalier-Smith (1982) and have extensive discussion of previous ideas, controversies, and evidence related to *Chlamydomonas* cell cycle control. Other recent articles that include substantial discussion of these issues but do not cite Cavalier-Smith (1982) include Vítová et al. (2011a and 2011b).

Literature review comments

Many of the brief summaries below converge the three points of what we call the "standard model": attainment of the first commitment depends on growth to a critical size, the time between first commitment and division falls within a fixed (but not particularly narrow) range, and the number of rounds of divisions is determined by parent cell size at the start of division. In many of these summaries, the issue of how the cells "decide" to initiate divisions at a particular

size/time is not directly mentioned. A fixed range of post-commitment times is often mentioned; if this is interpreted as hypothesizing that a fixed time separates commitment and divisions, then the issue of how cells "decide" to enter division is addressed indirectly. However, a fixed window of post-commitment times does not necessarily imply that divisions are initiated because a fixed time since commitment has elapsed. That is, these authors could be conveying an empirical observation that after ~5−10 h, the cells, for some unknown reasons, decide to initiate divisions. Therefore, it is not always clear whether these authors mean to imply that control of the decision to initiate divisions in *Chlamydomonas* is achieved by a sizer followed by a timer (as is explicitly hypothesized in Craigie and Cavalier-Smith (1982) and Vítová et al. (2011b)) or whether they simply mean to leave aside the issue of how a cell decides to initiate divisions. Some of these summaries do point out that divisions typically occur during the dark, implying that the duration of post-commitment growth is primarily determined by external factors. The brief summaries of *Chlamydomonas* cell cycle control that are considered below cite a small set of supporting papers (16 total).

Recent papers with brief verbal models of *Chlamydomonas* asexual cell cycle control

1) Rading MM, Engel TA, Lipowsky R, and Valleriani A (2011) Stationary size distributions of growing cells with binary and multiple cell division. *Journal of Statistical Physics* 145:1−22.

"Although many species are known to produce only two daughter cells, there are species, most notably algae like *Chlorella* and in particular *Chlamydomonas reinhardtii* that produce a larger number of daughter cells depending on the size of the mother cell at the time of division [Craigie and Cavalier-Smith 1982; Donnan and John 1983; Spudich and Sager 1980]. Since the time and size of cell division are stochastic variables, sister cells will, in general, produce a varying number of daughter cells. Experiments with the unicellular organism *Chlamydomonas reinhardtii* in synchronized cultures [Oldenhof et al. 2007] reveal that the timing and the division patterns depend on the growth conditions. On the other hand, the volume of the daughter cells at the time of release will strongly depend on the volume of the mother cell [Oldenhof et al. 2007; Oldenhof et al. 2006]."

2) Dillard SR, Van K, and Spalding MH (2011) Acclimation to low or limiting CO_2 in non-synchronous Chlamydomonas causes a transient synchronization of the cell division cycle. *Photosynthesis Research* 109:161-168.

"The CDC in Chlamydomonas includes a prolonged G-phase, following which the cells undergo two or more rounds of cell divisions involving alternating S (DNA synthesis) and M (mitosis) phases, with no additional, intervening G phases, to generate four or more daughter cells [Coleman 1982; Donnan and John 1983]. During the G-phase, passage through a transition state known as Commitment defines the point at which withdrawal of nutrients and/or light will not prevent the completion of at least one round of cell division [Donnan and John 1983; John 1987; McAteer et al. 1985; Spudich and Sager 1980]. Cells that have passed the Commitment transition will divide at least one time, but, regardless of whether they are provided with growth nutrients and light or not, cells remain in the G-phase for 5–8 more hours before entering the S and M phases [Craigie and Cavalier-Smith 1982; Donnan and John 1983; John 1984]. Under physiological conditions of alternating light and dark periods, Chlamydomonas cells generally become synchronized in such a way that growth occurs during the light period and the S and M phases occur during a fairly brief interval relatively early in the dark period [Donnan and John 1983; Spudich and Sager 1980]."

3) Olson, BJSC, Oberholzer M, Li Y, Zones JM, Kohli HS, Bisova K, Meisenhelder J, Hunter T, and Umen JG (2010) Regulation of the *Chlamydomonas* cell cycle by a stable, chromatin-associated retinoblastoma tumor suppressor complex. *The Plant Cell* 22:331−3347.

"*Chlamydomonas* uses a variation of the mitotic cell cycle, termed multiple fission, in which cells undergo a prolonged G1 period that is followed by multiple alternating rounds of S-phase and mitosis (S/M) to produce a uniform population of daughters (see Supplemental Figure 1 online) [Craigie and Cavalier-Smith 1982; Donnan and John 1983; John 1987; McAteer et al. 1985; Oldenhof et al. 2007; Spudich and Sager 1980] The extended G1 phase and rapid division cycles that characterize multiple fission are modifications that are critical for development and reproduction in plants and animals, making its investigation relevant for understanding both unicellular and multicellular cell cycles [Umen 2005].

MAT3/RB regulates the cell cycle at two key points. An early/mid G1 control point, termed Commitment, is passed when cells attain a minimum size that allows them to divide at least once. Cells that have passed Commitment remain in G1 for an additional 5 to 8 h, during which time they can continue to grow if conditions permit. At the end of G1, mother cells undergo one or more rounds of S/M to produce 2^n daughter cells. The S/M size checkpoint couples cell division number to mother cell size such that larger mother cells undergo more rounds of S/M than do smaller mother cells, thus ensuring a uniform-sized population of daughters [Craigie and Cavalier-Smith 1982; John 1987; Umen and Goodenough, 2001; Matsumura et al. 2003b; Umen 2005] (see Supplemental Figure 1 online). Under physiological conditions of alternating light and dark periods (e.g., 14 h light/10 h dark), the *Chlamydomonas* cell cycle becomes highly synchronized with G1 occurring in the light phase and S/M taking place early in the dark [Bisova et al. 2005], and this natural synchrony is advantageous for isolating homogenous populations of cells at defined positions in the cell cycle."

4) Fang SC and Umen JG (2008) A suppressor screen in Chlamydomonas identifies novel components of the retinoblastoma tumor suppressor pathway." *Genetics* 178: 1295−1310.

"Chlamydomonas proliferates using a multiple-fission cell cycle (Figure 1) that partially uncouples growth and division. The multiple-fission cell cycle is characterized by a long G_1 period during which cells can grow many- fold in size. At the end of G_1, mother cells undergo one or more rapidly alternating rounds of S phase and mitosis (S/M) to produce 2^n daughters of uniform size. Two cell-size checkpoints are integrated into the cell cycle to maintain size homeostasis [Umen 2005]. In early/ midG_1, cells pass commitment (Figure 1), a checkpoint that governs subsequent completion of the cell cycle and requires cells to attain sufficient mass for at least one cell [Craigie and Cavalier-Smith 1982; John 1984; McAteer et al. 1985]. Cells that have passed commitment will divide at least one time even in the absence of further growth, whereas precommitment cells stay in a resting state if growth ceases. Although it is formally analogous to Start in budding yeast, the commitment size checkpoint in Chlamydomonas does not coincide with the initiation of S phase. In fact, cells that have passed commitment remain in G_1 for an additional 5–10 hr (the delay period) and will continue to grow during the delay period if conditions permit. A second difference from yeasts is that the Chlamydomonas commitment size threshold is largely insensitive to growth rates [John 1987]. Depending on culture conditions, cells in G_1 can grow as little as 2-fold or as much as 30-fold; therefore, the number of S/M cycles must be regulated to produce uniform-sized daughters. Indeed, the number of cell divisions undertaken by a mother cell is related to her size, such that larger mother cells divide more times than smaller mother cells, thus ensuring that daughter cell size distributions are largely invariant [Craigie and Cavalier-Smith 1982; Donnan and John 1983]. Because S/M can occur in the absence of concurrent growth, daughter cell size can be used as a direct measure of the cell-size checkpoint that operates during S/M to control cell division numbers [Umen 2005; Umen and Goodenough 2001]."

5) Vlcek D, Sevcovicová A, Sviezená B, Gálová E, and Miadoková E (2008) Chlamydomonas reinhardtii: a convenient model system for the study of DNA repair in photoautotrophic eukaryotes. *Current Genetics* 53: 1–22.

"*C. reinhardtii* cells normally grow to many times their original size during a prolonged G1 phase and then undergo the multiple alternating rounds of DNA replication and mitosis to produce the daughter cells of uniform size. In each cell cycle a cell must make two size-dependent decisions—(1) whether and (2) how many times to divide. First decision, termed "commitment", takes place in G1. It is equivalent to "start" in yeast or "restriction point" in mammalian cells, and it is defined as the point at which the withdrawal of nutrients and/or light will not prevent the completion of at least one round of cell division [Donnan and John 1983; Spudich and Sager 1980]. Following the commitment, the cells maintained in nutrients and light can continue to increase in size before the DNA replication and mitosis begin [Craigie and Cavalier-Smith 1982; Zachleder and Van de Ende 1992]. The second size-based control mechanism then ensures that mother cells undergo the correct number of S phases and mitoses (reproductive cycles) to produce daughter cells with uniform size distribution [Donnan and John 1983; Lien and Knutsen 1979]."

6) Fang SC, de los Reyes C, and Umen JG (2006) Cell size checkpoint control by the retinoblastoma tumor suppressor pathway. *PLoS Genetics* 2: 1565–1579.

"The *C. reinhardtii* multiple fission cell cycle is characterized by a long G1 period during which cells can grow in size by many fold. In early/mid G1, cells pass Commitment, a size checkpoint similar to Start in yeast or the restriction point in animal cells that gates passage through the remainder of the cell cycle. Cells that have passed Commitment will complete the cell cycle, even if subsequent growth is stopped by the withdrawal of light or nutrients, whereas pre-Commitment cells will return to a G0 resting state if growth is halted [Craigie and Cavalier-Smith 1982; Spudich and Sager 1980]. Different measurements in wild-type strains have determined that a minimum cell size must be attained in order to pass Commitment [Craigie and Cavalier-Smith 1982; John 1987; Umen and Goodenough 2001; Matsumura et al. 2003b]. Post-Commitment cells do not immediately initiate S phase, but instead remain in G1 for an additional 5 to 10 h, where they can continue to grow and then finally undergo a rapid series of (n) alternating S phases and mitoses $(S/M)_n$ to produce 2^n daughters. A critical aspect of size regulation occurs during S/M where mother cell size—which can vary over a wide range depending on growth conditions—controls the number of S/M cycles to produce a uniform size distribution of daughters [Craigie and Cavalier-Smith 1982; Donnan and John 1983]. Because S/M can occur in the absence of concurrent growth, daughter cell size can be used as a direct gauge of cell size checkpoint function [Umen 2005]."

7) Umen JG (2005) The elusive sizer. *Current Opinion in Cell Biology* 17: 435–441.

"*Chlamydomonas* is a haploid unicellular green alga whose cell division program has a long G_1 phase during which cells can enlarge up to 30-fold. G_1 is followed by a short division phase in which mother cells alternate rapid rounds of S phase and mitosis (S/M). The extent of cell division (i.e. the number of rounds of S and M) is tightly controlled by a sizing mechanism that ensures uniform daughter cell size [Craigie and Cavalier-Smith 1982; Donnan and John 1983] (Figure 2). Once the decision to enter the division phase has been made, no further growth is required; in fact when cells are synchronized with a diurnal light/dark regime, cell division occurs during the dark phase when cells cannot grow."

8) Bradley BA and Quarmby LM (2005) A NIMA-related kinase, Cnk2p, regulates both flagellar length and cell size in Chlamydomonas. *Journal of Cell Science* 118: 3317–3326.

"In *Chlamydomonas*, cells undergo a prolonged G1 phase during which they grow until they reach a threshold size when cells commit to at least one round of division [Pickett-Heaps 1975]. Subsequently, the cells undergo multiple rounds of rapidly alternating S and M phases, producing 2, 4, 8 or 16 daughter cells of equal size [Coleman 1982; Craigie and Cavalier-Smith 1982; Donnan and John 1983]. The number of divisions is determined by cell size at commitment. Very little is known about the regulation of commitment size in *Chlamydomonas*, except that cells with mutations in a retinoblastoma protein (RB) homologue, Mat3p, are small due to a decrease in commitment size and an increase in the number of rounds of division post commitment [Umen and Goodenough 2001]."

Appendix 3: Selected calculations

Table 2, row 2: No cost of higher n, benefit of higher n is switching time

As described in the text, we use the intrinsic rate of population increase as a proxy for evolutionary fitness. Population rate of increase is a function of expected offspring number and generation time. Here we assume all geneotypes have a viability of 1 so expected number of offspring is 2^n. Fitness is given by

$$r_{\text{pop}} = \frac{\ln(2^n)}{T}. \tag{A1}$$

Cells grow in volume exponentially according to the equation

$$V_{\text{f}} = V_0 e^{r t_{\text{grw}}}. \tag{A2}$$

Using $\frac{V_{\text{f}}}{V_0} = 2^n$ and re-arranging to solve for t_{grw} gives

$$t_{\text{grw}} = \frac{n \ln(2)}{r}. \tag{A3}$$

Time between divisions is given by

$$T = t_{\text{grw}} + n t_{\text{div}} + t_{\text{oth}}. \tag{A4}$$

Substituting for t_{grw} gives

$$T = \frac{n \ln(2)}{r} + n t_{\text{div}} + t_{\text{oth}}. \tag{A5}$$

Substituting for T in (A 1) equation and simplifying gives

$$r_{\text{pop}} = \left(\frac{1}{r} + \frac{t_{\text{div}}}{\ln(2)} + \frac{t_{\text{oth}}}{n \ln(2)}\right)^{-1}. \tag{A6}$$

Recall that $r = c_1 Q + c_2$, so that substitution can also be made. Cell fitness increases as n increases for all values of the other parameters. Thus, a non-zero switching time is a benefit of higher n (or a cost of lower n). When $t_{\text{oth}} = 0$, we get the case considered in Row 1 of Table 2, no cost or benefit of higher n.

Table 2, row 4: Cost of higher n is sigmoidal growth and benefit of higher n is switching time

As before,

$$r_{\text{pop}} = \frac{\ln(2^n)}{T}. \tag{A7}$$

Cell growth in this case follows a sigmoidal trajectory, which we model using a logistic function. The final volume of a cell is given by

$$V_{\text{f}} = \frac{V_0 e^{\rho t_{\text{grw}}}}{1 - \frac{V_0}{V_\infty} + \left(\frac{V_0}{V_\infty}\right) e^{\rho t_{\text{grw}}}}. \tag{A8}$$

Using $\frac{V_{\text{f}}}{V_0} = 2^n$ and re-arranging to solve for t_{grw} gives

$$t_{\text{grw}} = \frac{1}{\rho}\ln(2^n) + \frac{1}{\rho}\ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right) \tag{A9}$$

As before,

$$T = t_{\text{grw}} + n t_{\text{div}} + t_{\text{oth}}. \tag{A10}$$

Substituting for t_{grw} and returning to the fitness equation gives

$$r_{\text{pop}} = \left(\frac{1}{\rho} + \frac{1}{n\,\rho\,\ln(2)}\ln\left(\frac{V_0 - V_\infty}{2^n V_0 - V_\infty}\right) + \frac{t_{\text{div}}}{\ln(2)} + \frac{t_{\text{oth}}}{n\ln(2)}\right)^{-1}. \tag{A11}$$

When $t_{\text{oth}} = 0$, we get the case considered in Row 3 of Table 2, sigmoidal growth cost and no benefit of higher n.

A) B)

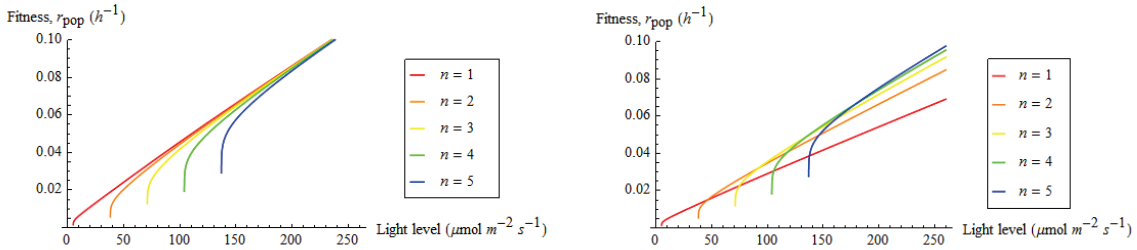


Figure A3 A) Fitness as a function of light level for different n values. Here $t_{\text{oth}} = 0$. Note that at all light levels, a cell would do better by having a lower n value. Other parameter values are $V_0 = 45$, $t_{\text{div}} = 1$, $c_1 = 0.00095$, $c_2 = 0.02155$, $c_3 = 81.4$, $c_4 = 0.021$. B) Fitness as a function of light level for different n values. Here $t_{\text{oth}} = 1$. Note that cells can do better by having higher n values, but only at high light levels. Other parameters are the same as in panel A.

Table 2, row 5: Cost and a benefit to higher n with colony-level effects on cell growth

The growth of a cell within a colony given by

$$V_{\text{g}}(t) = \frac{V_0 e^{\rho t}}{1 - \frac{V_0}{V_\infty/(a2^n + (1-a))} + \left(\frac{V_0}{V_\infty/(a2^n + (1-a))}\right) e^{\rho t}}, \tag{A12}$$

where a is a parameter that indicates how similar to unicellular growth the growth of a cell within a colony is. When $a = 0$, the cell within a colony grows exactly as a unicell would. Solving for growing time gives

$$t_{\text{grw}} = \frac{1}{\rho}\ln(2^n) + \frac{1}{\rho}\ln\left(\frac{V_0 - aV_0 + 2^n aV_0 - V_\infty}{2^n V_0 - a2^n V_0 + 2^{2n} aV_0 - V_\infty}\right), \tag{A13}$$

Fitness is given by

$$r_{\text{pop}} = \left(\frac{1}{\rho} + \frac{1}{n\,\rho\,\ln(2)}\ln\left(\frac{V_0 - aV_0 + 2^n aV_0 - V_\infty}{2^n V_0 - a2^n V_0 + 2^{2n} aV_0 - V_\infty}\right) + \frac{t_{\text{div}}}{\ln(2)} + \frac{t_{\text{oth}}}{n\ln(2)}\right)^{-1}. \tag{A14}$$

To see the effect of a on optimal n, refer to Figure 10.

APPENDIX D. DISTRIBUTIONS OF REPRODUCTIVE AND SOMATIC CELL NUMBERS IN DIVERSE *VOLVOX* (CHLOROPHYTA) SPECIES

Shelton, DE, AG Desnitskiy, and RE Michod. 2012. Distributions of reproductive and somatic cell numbers in diverse *Volvox* (Chlorophyta) species. *Evolutionary Ecology Research* 14:707–727.

Evolutionary Ecology Research, 2012, **14**: 707–727

Distributions of reproductive and somatic cell numbers in diverse *Volvox* (Chlorophyta) species

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ABSTRACT

Background: *Volvox* (Chlorophyta) asexual colonies consist of two kinds of cells: a large number of small somatic cells and a few large reproductive cells. The numbers of reproductive and somatic cells correspond directly to the major components of fitness – fecundity and viability, respectively. *Volvox* species display diverse patterns of development that give rise to the two cell types.

Questions: For *Volvox* species under fixed conditions, do species differ with respect to the distribution of somatic and reproductive cell numbers in a population of asexual clones? Specifically, do they differ with respect to the dispersion of the distribution, i.e. with respect to their intrinsic variability? If so, are these differences related to major among-species developmental differences?

Data description: For each of five *Volvox* species, we estimate the number of somatic and reproductive cells for 40 colonies and the number of reproductive cells for an additional 200 colonies. We sampled all colonies from growing, low-density, asexual populations under standard conditions.

Search method: We compare the distribution of reproductive cell numbers to a Poisson distribution. We also compare the overall dispersion of reproductive cell number among species by calculating the coefficient of variation (CV). We compare the bivariate (reproductive and somatic cell) dataset to simulated datasets produced from a simple model of cell-type specification with intrinsic variability and colony size variation. This allows us to roughly estimate the level of intrinsic variability that is most consistent with our observed bivariate data (given an unknown level of size variation).

Conclusions: The overall variability (CV) in reproductive cell number is high in *Volvox* compared with more complex organisms. *Volvox* species show differences in reproductive cell number CV that were not clearly related to development, as currently understood. If we used the bivariate data and tried to account for the effects of colony size variation, we found that the species that have fast embryonic divisions and asymmetric divisions have substantially higher intrinsic variability than the species that have slow divisions and no asymmetric divisions. Under our culture conditions, the Poisson distribution is a good description of intrinsic variability in reproductive cell number for some but not all *Volvox* species.

Keywords: cellular differentiation, development, eco-devo, evolution, multicellularity, reproductive allocation, robustness, *Volvox*.

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INTRODUCTION

The volvocine green algal genus *Volvox* includes about 20 species and offers an opportunity to study the evolution of cellular differentiation in a relatively simple system consisting of two kinds of cells, somatic and reproductive (Starr, 1970; Kirk, 1998; Desnitskiy, 2008). The asexual individual of *Volvox* is a spheroid in which thousands of small biflagellate somatic cells are arranged in a peripheral layer and are embedded in a transparent extracellular matrix (ECM). A colony also has far fewer (c. 1–20) asexual reproductive cells, known as gonidia. The numbers of reproductive and somatic cells translate directly into the major components of fitness – fecundity and viability, respectively.

Volvox and its unicellular relatives (e.g. *Chlamydomonas reinhardtii*) and less complex colonial relatives (e.g. *Pandorina*, *Eudorina*, *Pleodorina*), comprise a group known as the volvocine algae (reviewed in Coleman, 2012). Because of the grades of complexity apparent in existing species, volvocine algae reveal the ways in which multicellular development evolved during a unicellular-to-multicellular transition in the basic unit of evolution (Kirk, 2005; Herron and Michod, 2008). Volvocine algae can be found in diverse freshwater habitats such as deep permanent lakes and ephemeral pools. Despite their similar morphology, *Volvox* species comprise a polyphyletic genus, and unlike their smaller and less complex relatives, *Volvox* species demonstrate a striking diversity with respect to rate, diurnal rhythms, and light/dark control of gonidial divisions (Desnitski, 1995; Herron *et al.*, 2010). This diversity is simultaneously developmental and ecological, showing the potential of this small group for eco-devo research (Gilbert, 2001; Sultan, 2007).

Our focus is on one of the most basic aspects of *Volvox* development and fitness, the specification of differentiated reproductive and somatic cells during embryonic development. The evolution of specialized cell types is an innovation that creates a new challenge: the need for adaptive strategies of allocation to the distinct cell types. Species solve this problem, in part, through the evolution of mechanisms that specify particular numbers and sizes of cells during development. Solari and colleagues (2006) suggested that *Volvox* species with asymmetric divisions during development could have, because of their higher allocation to reproduction, an advantage in habitats that make motility less important, such as a shallow, transient pond. Thus they hypothesized a connection between development, mean reproductive allocation, and selective pressure.

Koufopanou (1994) has also addressed patterns of mean allocation in *Volvox*. With increases in total cellular volume of gonidia per colony among *Volvox* species, the total cellular volume of somatic cells per colony increases linearly (on a log-log scale) with a slope greater than one (Koufopanou, 1994). That is, larger (in terms of total cellular volume) *Volvox* species tend to invest proportionally less in reproductive cellular volume.

Here, we set aside issues of the mean level of reproductive allocation and focus on the dispersion of allocation outcomes that developmentally diverse *Volvox* species produce. We focus on variation that is produced under common genotypic and environmental conditions (as described, for example, in Larsen, 2005). Variation within a population that is apparently genetically and environmentally uniform has also been called micro-heterogeneity (Huang, 2009). [Here, we are concerned with apparently uniform populations of colonies, though Huang's (2009) terminology was developed in reference to cells.] If genetic and environmental conditions are truly uniform, then micro-heterogeneity must originate from within the individuals themselves. The root causes of micro-heterogeneity are at the biochemical level, and noisy gene expression is thought to be a major contributor to micro-heterogeneity (Ozbudak *et al.*,

2002; Blake *et al.*, 2003; Munsky *et al.*, 2012). It is important to keep in mind that stochasticity at the biochemical level (e.g. noisy gene expression) only generates higher-level phenotypic micro-heterogeneity by virtue of the interaction of many gene products. The nature of these downstream interactions (i.e. the gene regulatory networks) can affect the level of phenotypic noise (Munsky *et al.*, 2012). That is, it is possible for alleles to affect the level of phenotypic noise that results from the same level of noise in the underlying biochemistry. In the case of *Volvox* colonies, it is not necessarily noisy gene expression alone that could lead two colonies with the same genotype in the same environment to have slightly different levels of allocation to reproductive cells. The way the relevant gene products (with their slightly and randomly varying levels) interact in networks to produce allocation 'decisions' also could make a difference to the heterogeneity of outcomes in an otherwise uniform population.

Developmental systems evolve, in part, due to selection on the phenotypes they influence. If one of the effects of a developmental system is to influence the amount of heterogeneity in uniform populations, then this raises the question of whether natural selection has ever favoured (or disfavoured) particular developmental systems on the basis of this effect. *Volvox* is a particularly interesting study system in which to ask this question because the volvocine algae show the small, stepwise changes in development that occurred as the multicellular level of organization emerged. However, basic knowledge on the micro-heterogeneity produced by species with different developmental mechanisms is currently lacking for *Volvox*.

Here, we provide a detailed description of variation in cell-type numbers in developmentally diverse *Volvox* species. Because the cell numbers in a *Volvox* colony remain constant once embryonic cleavage is complete, simply counting each cell type in adult colonies provides information about the effects of embryonic cell-type specification (i.e. allocation) mechanisms. Allocation to specialized reproductive cells is a critical life-history trait in *Volvox* (Koufopanou, 1994; Solari *et al.*, 2006), thought to be affected by selection for an intermediate level of allocation (stabilizing selection). This study begins to make important connections between *Volvox* developmental features and aspects of the distributions of cell-type allocation found in populations of clones under uniform external conditions.

METHODS

The five *Volvox* species – *V. africanus* G.S. West (UTEX 2907), *V. aureus* Ehrenberg (UTEX 1899), *V. carteri* f. *weismannia* (Powers) Iyengar (UTEX 1876), *V. rousseletii* G.S. West (UTEX 1861), and *V. tertius* Meyer (UTEX 132) – used in this investigation differ in details of morphology and development (Table 1). We chose these species in order to encompass the major developmental diversity in this polyphyletic genus.

Desnitski (1995) categorized *Volvox* development into four major programs. We included species from three of the four programs in this study (Table 1). For two developmental programs, we included a pair of developmentally similar but non-sister species (program 2: *V. africanus*, *V. carteri* f. *weismannia*; program 4: *V. aureus*, *V. rousseletii*). The final species (*V. tertius*, program 3) is unusual in having slow, light-dependent divisions of large gonidia. We include *V. tertius* to capture more of the developmental diversity of *Volvox*. More species should be examined to help reveal large-scale among-species patterns, but here we focus on reporting detailed, within-species data that cover much of the developmental diversity of the genus.

Table 1. *Volvox* species included in this study

Species (developmental program)	Section	Clade	Developmental character states
V. africanus G.S. West (2)	*Merillospha era*	*Eudorina* group, clade A (forms a clade with *V. dissipatrix* and *V. tertius*)	• Large gonidia, no growth between fast and light-independent divisions • Occurrence of asymmetric divisions
V. carteri f. *weismannia* (Powers) M.O.P. Iyengar (2)	*Merillospha era*	*Eudorina* group, clade A (forms a clade with other *V. carteri* formas and *V. obversus*)	
V. tertius Meyer (3)	*Merillospha era*	*Eudorina* group, clade A (forms a clade with *V. africanus* and *V. dissipatrix*)	• Large gonidia, no growth between slow and light-dependent divisions
V. aureus Ehrenb. (4)	*Janetospha era*	*Eudorina* group, clade A (forms a clade with *Pleodorina californica* and *Pleodorina japonica*)	• Small gonidia, which undergo slow and light-dependent divisions • Cell growth between divisions • Cytoplasmic bridges in adult (thick in *V. rousseletii*; thin in *V. aureus*)
V. rousseletii G.S. West (4)	*Euvolvox*	Sect. *Volvox* (forms a clade with *V. barberi* and *V. globator*)	

Note: Sections are from Smith (1944) as modified by Kirk (1998). Clades are from Nozaki *et al.* (2006) and Nozaki and Coleman (2011). Developmental programs of asexual development are defined in Desnitski (1995) and Herron *et al.* (2010). Unfortunately, we were not able to include species with the first developmental program, such as *V. gigas*, *V. pocockiae*, and *V. spermatosphaera* (large and rapidly cleaving gonidia; no asymmetric divisions). The last common ancestor of colonial volvocines is thought to have had large mature asexual reproductive cells that divided rapidly and symmetrically, with little or no intervening growth and without the need for light. Cytoplasmic bridges did not persist to adulthood, and the asexual colony consisted of cells that were undifferentiated with respect to reproductive and somatic functions (Herron *et al.*, 2010).

We maintain the cultures in standard *Volvox* medium (SVM) (Starr and Zeikus, 1993) at 20°C and a diurnal light/dark (L/D) regime of 16 h/8 h (~60 μmol photons $\cdot m^{-2} \cdot s^{-1}$). We propagate the asexual colonies by adding a sub-sample of colonies to fresh media when the culture density becomes high (~700 colonies per millilitre). Genetic differences among individual colonies within a strain are due to new mutations that have arisen during culture maintenance; many of these mutations have likely become fixed due to the repeated population bottlenecks that occur upon transfer to fresh media. Therefore, we assume that individual colonies of a strain are genetically extremely similar.

For observations, we used the algae in a state of active asexual reproduction: 3–8 days after the transfer of a small number of colonies to tubes with 5 mL of SVM. These culture

conditions are favourable enough for all species studied to undergo population growth, and we always sampled spheroids from a growing (not overcrowded) population. Although *V. carteri* f. *nagariensis* is often cultured at 30°C, this culture condition is anomalous compared with the commonly used culture temperature of other species of *Volvox*. We did not use *V. carteri* f. *nagariensis*, and used 20°C because it is known to be a suitable temperature for culturing a range of *Volvox* species (Darden, 1966; Kochert, 1968; McCracken and Starr, 1970; Herron *et al.*, 2010). We used only 5 mL of media because only a small number of spheroids was required and because lack of aeration is not a problem in such a small volume (Yates *et al.*, 1975).

We scored the numbers of somatic and reproductive cells of the colonies fixed with iodine for 40 colonies of each species. We also made separate additional rapid counts of gonidial numbers (without accompanying counts of somatic cells) using 200 colonies of each of the five species of *Volvox*. We selected colonies randomly with respect to gonidial and somatic cell number. We based most observations on microphotographs that were taken with a digital camera (Moticam 2300, Motic Industries, China). For some observations, we recorded cell number directly from observations under a microscope (magnification 400×). As a rule, we used young colonies with unicellular gonidia in the period of growth. In the cases of *V. aureus* and *V. rousseletii*, in which it is not always possible to distinguish unicellular gonidia in the young colonies, we also used the colonies with embryos in the early cleavage (2- to 16-celled stages). It was not difficult to count the small number of gonidia per colony but determining the number of abundant somatic cells was not so easy. It is possible to estimate the total number of cells forming a colony using the method devised by Janet (1912) and later used by Pocock (1933) and Starr (1969), i.e. multiply the square of the number of cells on a great circle of the colony by 0.367. (A great circle is a circle on the surface of a spheroid that surrounds it at its largest diameter.) The somatic cell count is, of course, an estimate only. We did not subtract the small number of gonidia from the huge total cell number.

We developed a model of the major processes that gave rise to our observed cell counts. We simulated the model under various levels of developmental error and size variation. For each simulated dataset, we calculated a 95% confidence ellipse describing the region occupied by the data. We also calculated the slope of the standardized major axis (SMA) line for each simulated dataset for comparison with the observed data. We used the Multivariate Statistics Package in Mathematica 8 to estimate the 95% confidence ellipse. We calculated the slope of the standardized major axis by taking the geometric mean of the slope of the regression of $\log_{10}$(somatic cell number) on $\log_{10}$(gonidial cell number), and the slope of the regression of $\log_{10}$(gonidial cell number) on $\log_{10}$(somatic cell number) (Smith, 2009). Each simulated dataset initially had the same number of colonies as the observed data ($n = 40$). We constrained the simulated number of somatic and gonidial cells to be greater than zero by discarding simulated data points for which these conditions were not met. We repeated the simulation for each of 50 combinations of parameters 50 times for a total of 2500 simulated confidence ellipses per species.

We compared the simulated and the observed confidence ellipses and standardized major axis slopes. The centroid of the data changed very little with our simulations, so we did not analyse this aspect of the data. For each simulated ellipse, we took the sum (over the two radii) of the absolute value of the difference between the radius from the observed data and the radius from the simulated data. To compare the slope of a line that fits the data, we calculated the absolute value of the difference between the SMA slope of each simulated dataset and the observed data.

Statistical analyses were conducted in R (R Development Core Team, 2012) and Mathematica 8. Mathematica 8 was used to produce simulated datasets.

RESULTS

For each species, gonidia were counted in 200 colonies. The gonidia census values from the gonidia-soma dataset were not included in the gonidia-only dataset because these colonies were grown at a different time and could have introduced unwanted differences in environmental conditions (e.g. a different batch of growth medium). We compare the gonidia-only data to a Poisson distribution (Fig. 1). The data from *V. africanus* and *V. rousseletii* fit a Poisson distribution well (chi-squared goodness-of-fit test, $P > 0.05$). *Volvox carteri* f. *weismannia* and *V. tertius* both appear to have negatively skewed distributions of gonidial counts and lower variance than would be expected for a Poisson distribution. *Volvox aureus* appears to have a bimodal distribution of gonidial counts (Fig. 1).

The mean and standard deviation of gonidia per colony are correlated for these five species (correlation coefficient = 0.423). Therefore, we calculated the coefficient of variation for each species to obtain a normalized measure of the dispersion of gonidial numbers. We used jackknifing to estimate the standard error of the CV. Because gonidial cell number was not normally distributed (Fig. 1), we also calculated a robust version of the CV for comparison (Hoaglin *et al.*, 1983). Species differ with respect to gonidial cell number CV – in other words, in their precision of germ cell specification under these conditions (Fig. 2). The species with the highest gonidial cell number CV (*V. rousseletii* and *V. africanus*) do not share a unique set of developmental traits and are not closely related. Note that the *V. aureus* CV in particular should be interpreted with caution because this species' bimodal distribution of gonidial cell counts (Fig. 1) makes the CV less useful as a measure of dispersion.

Our data on somatic and reproductive (gonidial) cell numbers in five *Volvox* species are summarized in Table 2. The literary data on somatic and reproductive cell numbers in the same species of *Volvox* (Desnitskiy, 2008) are represented in parentheses. Three of the five species had similar mean somatic cells per colony (*V. africanus*, *V. carteri* f. *weismannia*, and *V. aureus*; Table 2). *Volvox tertius* has a relatively low mean and standard deviation of somatic cell number. *Volvox aureus* appears to have a bimodal distribution of somatic cell numbers (not shown). *Volvox tertius* and *V. aureus* had about 8 gonidia per colony, whereas *V. africanus* and *V. carteri* f. *weismannia* (the species with developmental program 2) had lower mean numbers of gonidia per colony. Compared with the other species, *V. rousseletii* had a higher mean number of somatic cells and an intermediate mean number of gonidia (Table 2).

In *Volvox* species, colonies are larger in better environments. We measured the CV of gonidial cell number per colony (Fig. 2) for colonies grown under one set of external conditions in order to estimate the within-species variation in allocation to gonidial cells, given roughly equivalent size of colonies within a species. However, it is possible that small differences among colonies in external conditions created within-species variation in colony size, and thus contributed to the observed CV of gonidial cells per colony. Our aim is to understand the variation in gonidial cell number that is due to intrinsic variability. So variation in gonidial cell number that results from variation in colony size is a potential confounding factor. We addressed this issue by examining both gonidial and somatic cell number for 40 colonies of each species. Within all species, gonidial and somatic cell

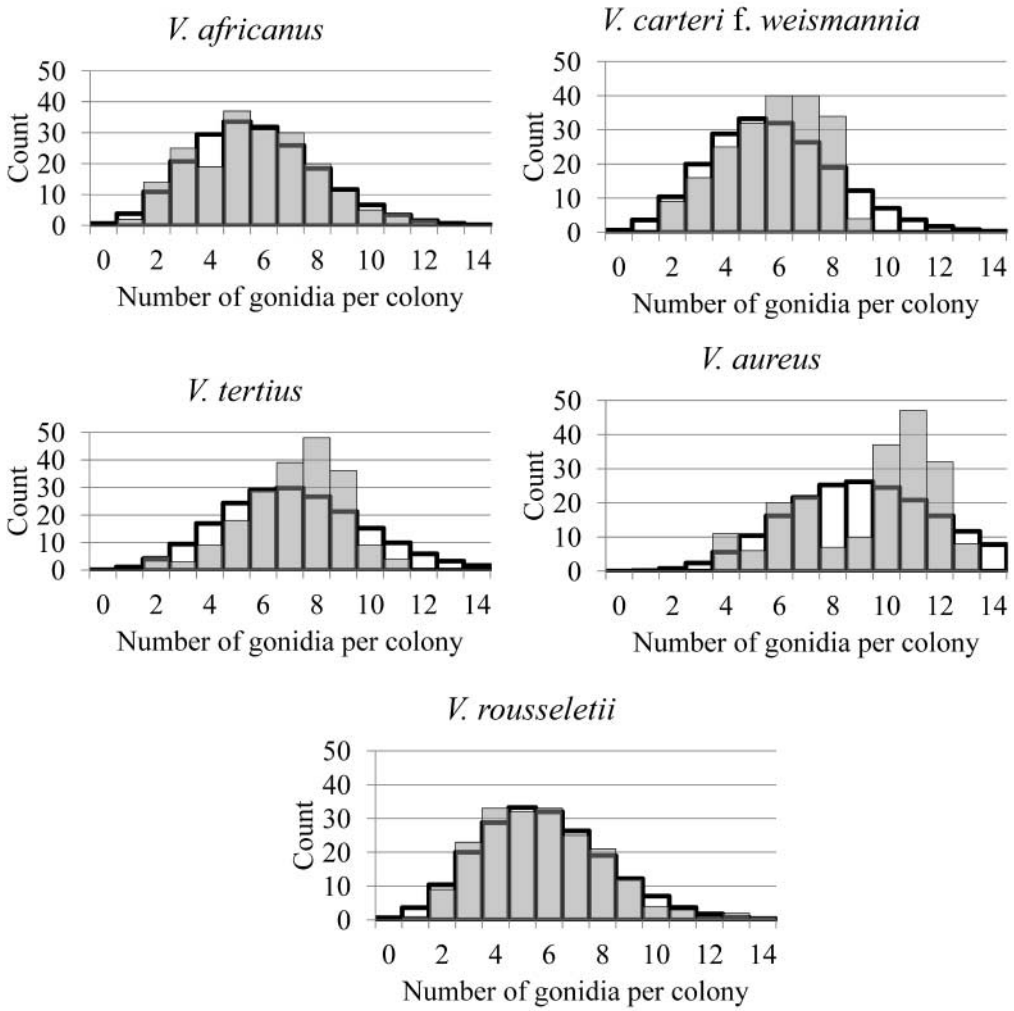


Fig. 1. Histograms showing the count of colonies that have a particular number of gonidia ($n = 200$ colonies per species). Grey bars are the observed data; white bars are the expectations based on the Poisson distribution. Maximum likelihood was used to estimate λ for each species and a chi-squared goodness-of-fit test was implemented using the vcd package in R. *V. africanus*: $\lambda = 5.696$, $P = 0.258$; *V. carteri* f. *weismannia*: $\lambda = 5.77$, $P = 1.3 \times 10^{-11}$; *V. tertius*: $\lambda = 7.17$, $P = 1.9 \times 10^{-12}$; *V. aureus*: $\lambda = 9.345$, $P = 2.4 \times 10^{-23}$; *V. rousseletii*: $\lambda = 5.77$, $P = 0.605$.

number were positively correlated (Fig. 3), a pattern best explained by substantial variation in colony size (Van Noordwijk and De Jong, 1986). The positive gonidial-somatic cell number correlations indicate that the assumption of negligible variation in colony size could be problematic. Thus, we consider the interpretation of gonidia-only CVs (Fig. 2) in terms of intrinsic variability to be preliminary.

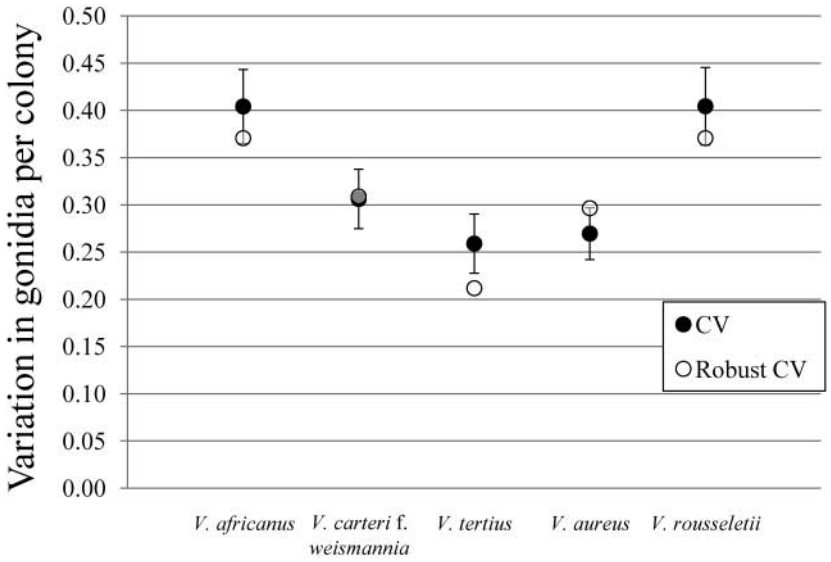


Fig. 2. Estimates of the coefficient of variation (CV) of gonidial cells per colony for each species. Black circles show the CV (sample standard deviation divided by sample mean). Error bars show ± 2 standard errors. Standard error of the CV was estimated by jackknifing (using R). Because the distributions of gonidial cells were generally not normally distributed (Fig. 1), we also calculated a robust version of the CV for comparison. White circles show a robust version of the CV, the F-pseudosigma (Hoaglin *et al.*, 1983, p. 40) divided by the median.

MODEL

To understand the effects of colony size variation, we model two major processes that contribute to the variation in our bivariate cell count distributions: allocation of mass to discrete cell types during embryonic development, and increases in allocation to reproduction in response to increases in total mass. The purpose of the model and simulations is to find values of intrinsic variation (i.e. error) that are consistent with our data, given a range of (unknown) levels of variation in size. We specified our model parameters so as to describe expected distributions of cell counts, but otherwise our model does not differ from the model developed by Van Noordwijk and De Jong (1986). Parameters are described in Table 3.

Two conceptually separate allocation strategies occur during the lifetime of a *Volvox* asexual colony. The mass of the parental gonidium (plus any mass produced by growth during embryonic development in some species) is divided into particular numbers and initial sizes of each cell type. The cell-type number is then fixed, although allocation 'decisions' continue through differential growth of gonidia and somatic cells. The first phase of allocation is closely connected to mechanisms of embryonic development, so that is our focus here and our reason for focusing on the mass of cells at the end of embryonic divisions. The total cellular mass of a colony (in units of somatic cells) at the end of embryonic divisions is the sum of the number of somatic cells and the number of gonidial cells multiplied by the ratio of gonidial cell mass to somatic cell mass (r). Not all colonies have the same mass, so M_i can differ from $\bar{M}$:

$$M_i = s_i + rg_i. \quad (1)$$

Table 2. Cell numbers by cell type for asexual colonies of five species of *Volvox*

	Somatic cells per colony		Gonidia per colony	
Species	Estimated range compared with literature, $n = 40$ colonies	Mean ± S.D., $n = 40$ colonies	Estimated range compared with literature, $n = 240$ colonies	Mean ± S.D., $n = 40$ colonies
V. africanus	1366–4522 (2300–6000)	2390 ± 778	1–12 (2–8)	5.0 ± 1.7
V. carteri f. *weismannia*	587–5024 (1400–6000)	2250 ± 820	2–9 (6–12)	5.5 ± 1.7
V. tertius	530–1747 (500–2000)	1180 ± 292	2–11 (2–12)	8.0 ± 1.6
V. aureus	1070–3894 (200–3200)	2650 ± 788	3–13 (4–15)	8.0 ± 2.7
V. rousseletii	3106–12,971 (14,000–42,000)	7449 ± 2701	1–14 (1–20)	6.4 ± 2.7

Note: The number of somatic cells was estimated by squaring the number of somatic cells observed in the great circle and multiplying by 0.367. Literature data on somatic and reproductive cell numbers in the same species of *Volvox* (summarized in Desnitskiy, 2008) are in parentheses (Darden, 1966; Iyengar and Desikachary, 1981; Janet, 1912; Kochert, 1968; McCracken and Starr, 1970; Nozaki, 1988; Smith, 1944; Solari, Kessler, and Michod, 2006). In general, our data are in accord with those from the literature, although some differences deserve attention. First, the same *V. aureus* strain was investigated by Darden (1966) but he dealt with colonies consisting of only 500–1000 cells, whereas colonies in our study had a median estimated value of 2940 somatic cells. In the colonies of the same strain of *V. rousseletii*, McCracken and Starr (1970) reported many more cells (usually 20,000–30,000 vs. our median somatic cell number of 7558). By contrast, Solari and colleagues (2006) recorded only about 3000 somatic cells in the same strain. It is problematic to compare our data on *V. carteri* f. *weismannia* with Kochert's (1968) work because he used another strain of the same species and form of *Volvox*.

Table 3. Description of model parameters

	Description
A	Proportion of the total cellular mass of a colony that is allocated to gonidial cells. Random variable drawn from a normal distribution with mean of $\bar{A}$ and a standard deviation of $s.d._A$. $\bar{A}$ is assumed to be the 'target' of the developmental system, so $s.d._A$ corresponds to intrinsic variability. $0 < A < 1$
g	Number of gonidial cells per colony
M	Cellular mass of a colony. Random variable drawn from a normal distribution with mean $\bar{M}$ and a standard deviation of $s.d._M$
r	Ratio of the mass of a prospective gonidium and the mass of a prospective somatic cell at the end of embryonic cell divisions. $r \geq 1$
s	Number of somatic cells per colony

The total initial mass of gonidial cells per colony (rg_i) increases as the total size of the colony (M_i) increases. That is, colonies living in better external conditions make more gonidial cells. Here, we consider the total gonidial mass to be a constant fraction (A) of the total mass of the colony. Although a relationship in which proportionally less is allocated to gonidia as total size increases has been described among *Volvox* species (Koufopanou, 1994), we do not incorporate that allometry here. Instead, we assume that the allocation fraction (A) is independent of total size (M). Our focus here is on the relationship within a *Volvox*

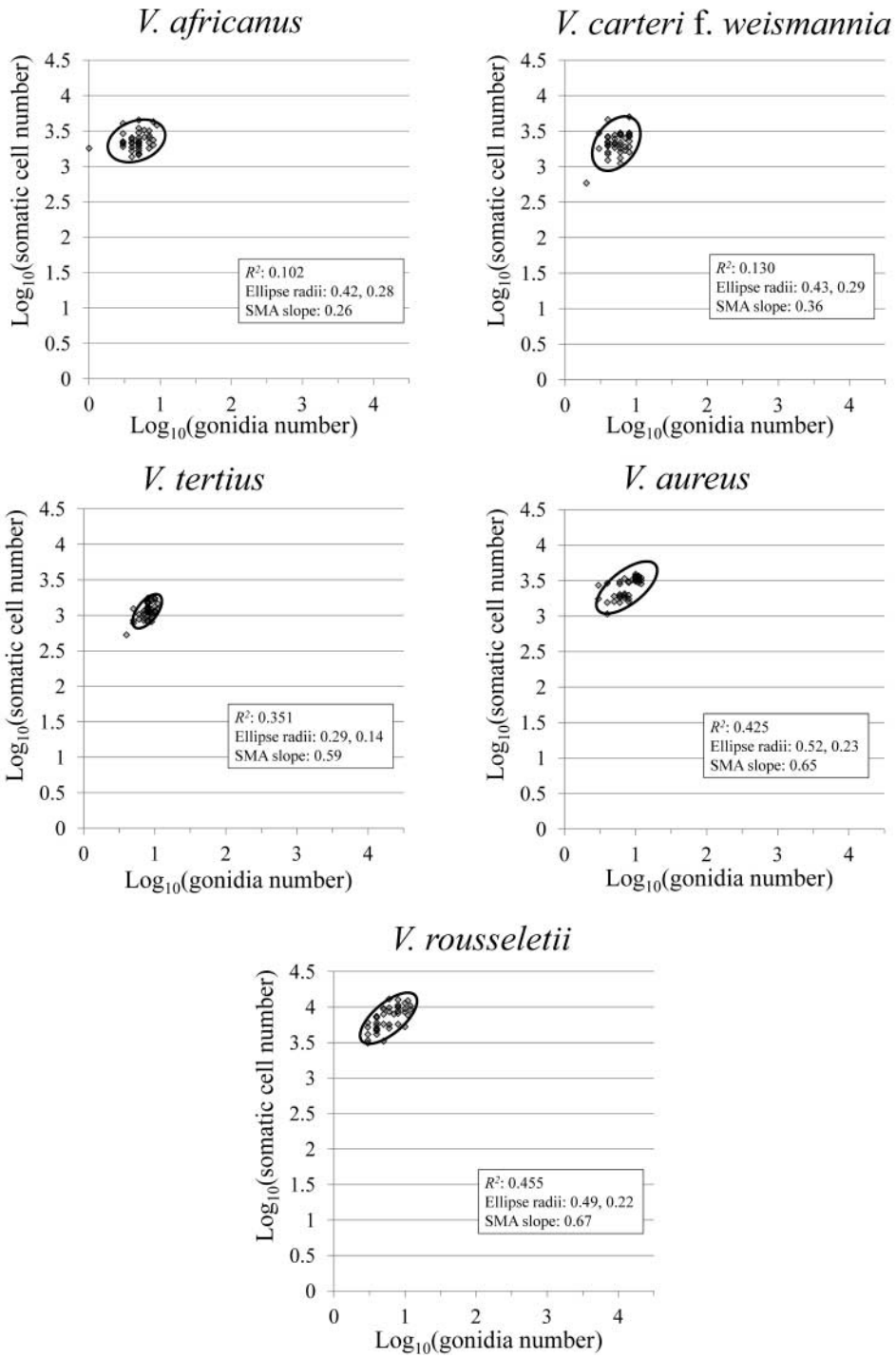


Fig. 3. Relationship between log-transformed gonidial cell number and somatic cell number for five species of *Volvox*. Each point is one colony ($n = 40$) and the ellipse is a 95% confidence ellipse. The R^2, radii of the ellipse semi-axes, and slope of a standardized major axis best-fit line are also given.

species, which could be different from the between-species pattern. Also, although the variation in colony size (M) under our conditions appears to be non-negligible, the scale of differences in size is still small compared with the interspecific comparisons of Koufopanou (1994). Van Noordwijk and De Jong (1986) also assumed that allocation fraction is independent of total size (acquisition, in their terms). Because development is not perfect, the realized allocation to gonidial mass (A_i) can deviate from the 'target' allocation ($\bar{A}$):

$$rg_i = A_i M_i. \tag{2}$$

We assume that both A and M are random variables drawn from a normal distribution. The variation in allocation ($s.d._A$) corresponds to intrinsic variation (or developmental error), and is the main parameter we would like to estimate from the data. From equations (1) and (2), we can express our expectation of the number of somatic cells per colony and the number of gonidial cells per colony in terms of the parameters in Table 3:

$$g_i = \frac{1}{r}(A_i M_i), \tag{3}$$

$$s_i = (1 - A_i) M_i. \tag{4}$$

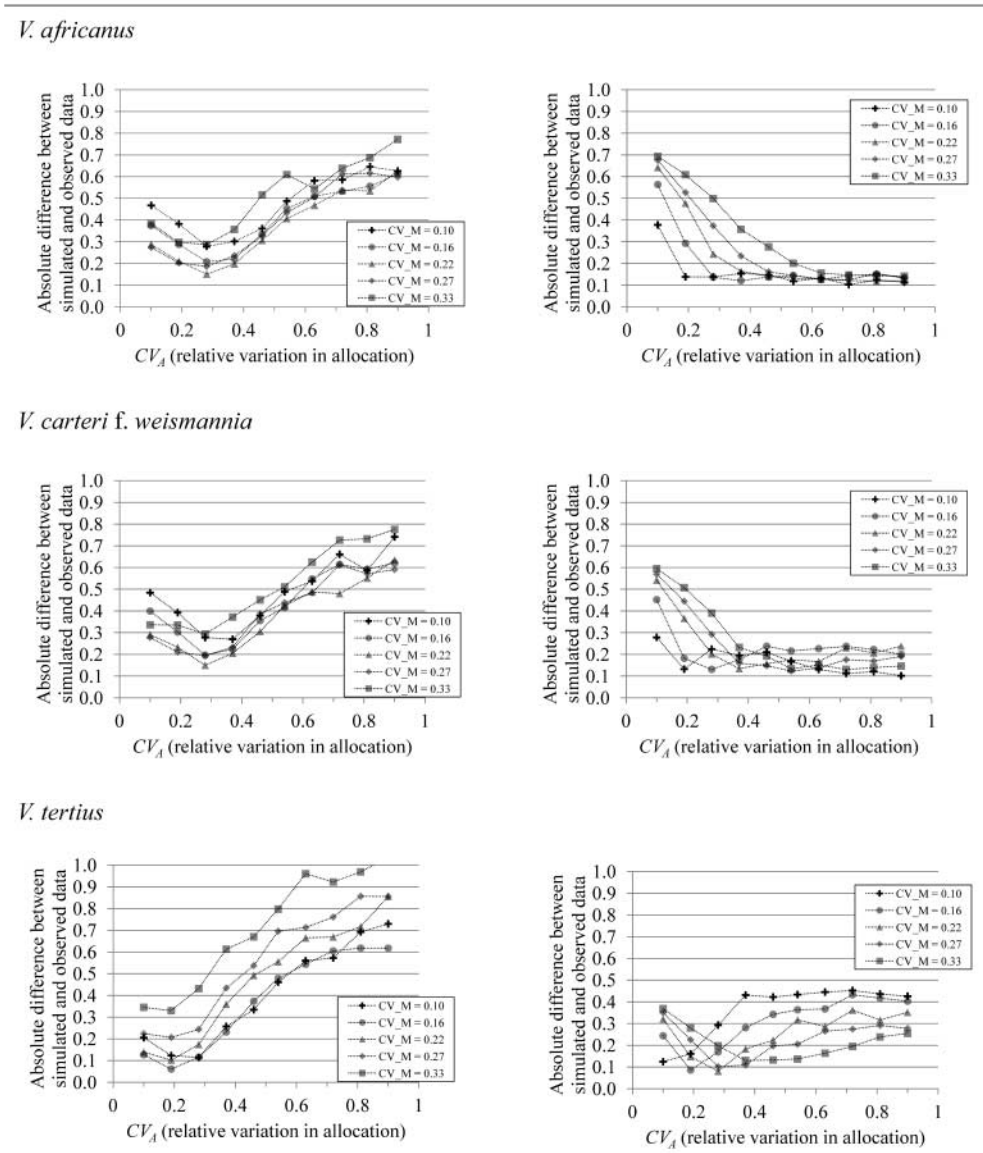
For each species, we estimated $\bar{M}$ and $\bar{A}$ from the cell count data combined with a rough estimate of r (Table 4). Although it would be ideal to have estimates of $\bar{M}$ and $\bar{A}$ that are independent of our cell count data, we do not believe this to be a major problem. Both developmental error and size variation are likely to be symmetrical or nearly so. Therefore, our data probably give a reasonable estimate of the mean size and mean allocation for each species under our conditions. Note that this model ignores integer effects that actually constrain s_i and g_i and ignores measurement errors in our observations of s_i and g_i. We also ignore the mass of the extracellular matrix.

Using the species-specific estimates of r, $\bar{M}$, and $\bar{A}$, we simulated datasets using differing values of $s.d._A$ and $s.d._M$. We chose $s.d._A$ and $s.d._M$ so that the CV of A and the CV of M

Table 4. Species-specific parameter estimates

Species	r	$\bar{M}$	$\bar{A}$
V. africanus	32	2439	0.0610
V. carteri f. *weismannia*	32	2281	0.0738
V. tertius	1	1154	0.0136
V. aureus	1	2541	0.0059
V. rousseletii	2	6965	0.0017

Note: The ratio of the mass of a gonidium and the mass of a somatic cell at the end of embryonic cell divisions, r, has not been reported for most *Volvox* species. We assumed that mass and volume are directly proportional and used the *V. carteri* f. *nagariensis* value of 32 (Kirk, 1998, p.137) for both of the species that have asymmetric cell divisions during development. For *V. rousseletii*, we used $r = 2$, again based on the cell volume (Kirk, 1998, p. 34). For *V. aureus* and *V. tertius*, the prospective gonidia are approximately the same volume as the prospective somatic cells at the end of embryonic cleavage (Darden, 1966; Ireland and Hawkins, 1981; Desnitskiy, 2008), so we used $r = 1$. For each colony, we estimated $\bar{M}$ and $\bar{A}$ by taking the germ (g_c) and soma (s_c) number from the centroid of the data. For $\bar{M}$ we used $rg_c + s_c$ and for $\bar{A}$ we used $rg_c/(rg_c + s_c)$. These parameter estimates were used, along with varying values of $s.d._A$ and $s.d._M$ to produce the simulated datasets.



varied consistently across species. That is, we looked across the same range of relative variation in allocation and size for each species. As described in the Methods, we compared the observed and simulated datasets based on the similarity of the radii of the 95% confidence ellipse and based on the slope of the SMA line. To find combinations of $s.d._A$ and $s.d._M$ that are most consistent with our data, we plotted the mean (of 50 simulations) of each similarity metric (radii and slope) for each combination of $s.d._A$ and $s.d._M$. We present lines of constant $s.d._M$ to emphasize the effect of changing $s.d._A$ (when all else is equal) on the similarity of the simulations to the data (Fig. 4).

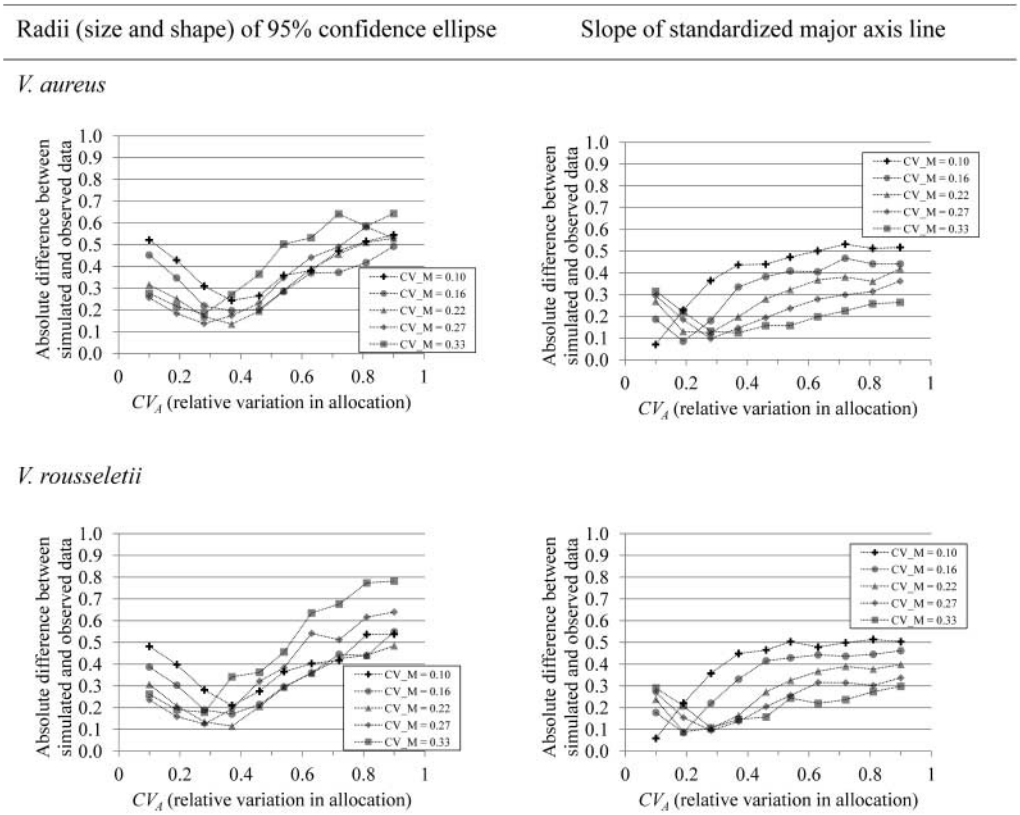


Fig. 4. The similarity of the simulated and observed data is shown according to two metrics: radii of 95% confidence ellipses (left column) and slope of SMA line (right column). Each data point represents the mean of 50 simulations with a particular combination of parameter values. The CV_A values used are: 0.10, 0.19, 0.28, 0.37, 0.46, 0.54, 0.63, 0.72, 0.81, and 0.90. The CV_M values are indicated in the figure legend. The $s.d._A$ value that was used for the simulation is the CV_A times the species-specific $\bar{A}$ (Table 4) and likewise for $s.d._M$. For the similarity of the radii of the 95% confidence ellipses (left column), we fit a polynomial curve (order 3) to the pooled data to get a rough estimate of the value of CV_A for which the difference between the data and simulations was the lowest. This value of CV_A is: *V. africanus*, 0.26; *V. carteri* f. *weismannia*, 0.32; *V. tertius*, 0.11; *V. aureus*, 0.31; *V. rousseletii*, 0.29.

As discussed below, the model analysis shows that approximately the same relative level of variation in allocation (i.e. CV_A) is most consistent with the radii of the observed confidence ellipse for four of the five species. Data from the fifth species, *V. tertius* (program 3), were most consistent with a substantially lower relative variation in allocation. In terms of absolute variation (i.e. $s.d._A$), program 2 species (*V. africanus* and *V. carteri* f. *weismannia*) show substantially higher values than the other species (*V. tertius*, *V. aureus*, and *V. rousseletii*). The model analysis also shows that the slope of a line fitted to the data is not very informative about CV_A in the absence of information about CV_M. Nevertheless, the slopes of lines fitted to the *V. africanus* and *V. carteri* f. *weismannia* data appear inconsistent with very low values of CV_A, whereas very low values of CV_A are not ruled out (based on slope alone) for the other species.

DISCUSSION

Non-random patterns in *Volvox* cell type distributions

A developmental system can affect the distribution of outcomes for clones raised in one macro-environment in at least two different ways. First, the developmental system can affect the overall level of dispersion of the phenotypes, i.e. the level of random deviations from the target phenotype. We consider this effect in the subsection that follows this one. Second, the developmental system can cause systematic changes in the likelihood of particular outcomes. We now discuss this second type of effect, which is known as 'developmental constraint' or 'generative bias' (Maynard Smith *et al.*, 1985; Brakefield, 2006).

Volvox asexual development consists of rounds of synchronous cell divisions, and the differentiation of cells during these divisions has been particularly well studied in *V. carteri* f. *nagariensis* (summarized in Kirk, 2001). For this species, the somatic vs. gonidial cell fate has been linked to a threshold cell size at the end of embryonic divisions (Pall, 1975; Kirk *et al.*, 1993). Size variation in *V. carteri* f. *nagariensis* is generated primarily by divisions in which the offspring cells are of substantially different sizes (asymmetric divisions). Final somatic cell number in this species depends on four developmental parameters: (1) The round of divisions at which asymmetric divisions begin, typically round 6 under favourable conditions. Previous literature disagrees on whether asymmetric divisions can sometimes begin at different rounds of division within a single embryo (Gilles and Jaenicke, 1982; Kirk, 1998, p. 136). (2) The number of cells that undergo asymmetric divisions when asymmetric divisions begin, typically 16 under favourable conditions. (This also equals the number of gonidia in the final colony.) (3) The number of rounds of asymmetric divisions. Each asymmetric division of a gonidial initial maintains the existence of that gonidial initial and gives rise to a somatic initial, capable of undergoing symmetric divisions. Green and Kirk (1981) suggest that in their cultures, this number was 4 (i.e. the initial asymmetric divisions of 16 undifferentiated cells at round 6, then three additional rounds of asymmetric divisions of gonidial initials). (4) The total number of synchronous cell divisions in the somatic cell lines. Green and Kirk (1981) suggest this number is the same for somatic cell lines that originated from an asymmetric division and those that have a history of only symmetric divisions. Different total rounds of division in these two cell populations would complicate the picture.

Green and Kirk (1981) reported that *V. carteri* f. *nagariensis* cultures in their conditions fell into two discrete categories of somatic cell number: colonies with ~2000 somatic cells and colonies with ~4000 somatic cells. They attribute this difference to colonies that underwent either 11 or 12 rounds of cell division in the somatic cell lines (i.e. to variation in the fourth developmental parameter). Intra-colony variation in the total number of rounds of divisions, which would produce intermediate-soma-number colonies, was apparently absent or very rare. In our study, we found evidence for two discrete soma number categories in *V. aureus*, but not in the other species observed (Fig. 3). This result could be due to developmental constraint. By this interpretation, the optimal phenotypic target (somatic cell number that would be best, given our conditions) is towards the middle of our observed values, and the *V. aureus* developmental system constrains the actual phenotypes to tend to fall on either side of the target.

Non-random patterns of gonidial cell number (and location) under uniform external conditions have also been reported for *V. carteri* f. *nagariensis*. Wenzl and Sumper (1979)

showed a distinct even–odd effect; colonies with even-numbered gonidia were much more common than colonies with odd-numbered gonidia in their conditions. Gilles and Jaenicke (1982) reported a distribution with sharp cut-offs (few colonies below 8 or above 16) and with apparently depressed numbers of observations in categories other than 8, 12, and 16 gonidia per colony. They suggested that the patterns they observed could be explained if, within a colony, some cells began dividing asymmetrically at division 5 whereas others began dividing asymmetrically at division 6 (Gilles and Jaenicke, 1982). However, this explanation has been disputed (Kirk, 1998, p. 136).

Rather than being a sharp lower bound, 8 gonidia per colony appears to be a relatively sharp upper bound for the *V. carteri* f. *weismannia* gonidia-per-colony distribution. Similarly, 9 gonidia per colony may be a relatively sharp upper bound for *V. tertius* in our culture conditions (Fig. 1). Both the *V. tertius* and *V. carteri* f. *weismannia* data did not match the Poisson distribution well. *Volvox aureus* also had a distribution that looked very different from the Poisson distribution, although in this case the primary difference is the apparent bimodality in the observed data. By contrast, both *V. africanus* and *V. rousseletii* matched the Poisson distribution well (Fig. 1).

The mechanistic sources of these features of the gonidia-per-colony distributions remain obscure. The Poisson distribution is a reasonable null model for the distribution that one would expect in a uniform external environment and in the absence of developmental constraints. We hope to see further study into the mechanistic basis for constraints in the species that differ from the Poisson distribution (*V. aureus*, *V. carteri* f. *weismannia*, and *V. tertius*). Additionally, these data highlight the substantial between-species differences in variational properties that exist in *Volvox* despite more superficial similarities in morphology.

Dispersion of cell type numbers of *Volvox* clones in one environment

We now turn to the other way in which a developmental system can affect the distribution of outcomes, i.e. through determination of the overall level of dispersion around a target outcome. In our counts of gonidial cell number per colony, *V. tertius* stands out as having low levels of dispersion compared with the other species (Fig. 2). However, this result should be explored further under other conditions to discover how robust it is, particularly because there is no standard methodology for comparing levels of dispersion among species and because the use of CV as a measure of dispersion has important limitations (Jacobs and Podolsky, 2010).

All species had a positive correlation of $\log_{10}$-transformed gonidial cell counts and $\log_{10}$-transformed somatic cell counts (Fig. 3). The positive gonidia-soma correlations probably indicate that there was non-negligible variation among colonies within a species in colony size and low variation among colonies within a species in reproductive allocation, i.e. cell type specification (Van Noordwijk and De Jong, 1986). The colony size variation could be due to colonies acquiring different amounts of resources in different micro-habitats or to maternal effects in which colonies that originate from a specific part of the mother colony have better conditions. Either way, the ultimate origin of size variation in our population is likely to be external to the colonies. Therefore, accounting for the effects of size variation is necessary to estimate the level of intrinsic variation in each of our populations.

To better understand the effects of colony size variation within a species, we developed a model of the fundamental factors thought to underlie our bivariate cell count observations

(equations 1 and 2). To isolate the effect of intrinsic variation ($s.d._A$), we would need to know the average and standard deviation of colony size ($\bar{M}$ and $s.d._M$) as well as the average initial ratio of gonidial to somatic cell mass (r) and the average (i.e. target) proportion of mass initially allocated to gonidia in that environment ($\bar{A}$). Here we assume that the scaling exponent (to which M_i is raised in equation 2) is simply unity, although independent information on this would also be useful. We used the literature for a rough estimate of r and used our data (with the r for each species) to estimate $\bar{M}$ and $\bar{A}$ (Table 4). This still left us, however, with the unknown confounding factor of variation in colony size ($s.d._M$). We explored the effects of a wide range of $s.d._M$ and $s.d._A$ values with simulations that used the species-specific values for the other parameters. For the observed data and the simulated data, we describe the bivariate dataset using a 95% confidence ellipse and the slope of a SMA line. An ellipse is a convenient way to describe the data even though we do not think that the actual shape underpinning the dispersion of the data is an ellipse. Under our model, the region in which the data are expected to fall is trapezoidal (or even almost triangular under some conditions) (Van Noordwijk and De Jong, 1986).

Our model analysis of the bivariate data suggests some interesting patterns in the among-species comparisons. With respect to differences between the observed and simulated data in the radii of the ellipse (left column, Fig. 4), a similar level of CV_A is most consistent with the data for four of the five species, under all of the values of CV_M examined. *Volvox africanus*, *V. carteri* f. *weismannia*, *V. aureus*, and *V. rousseletii* all have simulations that are most similar to the data at roughly $CV_A = 0.29$. This indicates drastically different values of $s.d._A$ for *V. africanus* and *V. carteri* f. *weismannia* (~0.016 and ~0.023, respectively) versus those for *V. aureus* and *V. rousseletii* (~0.0018 and ~0.0005, respectively). The fifth species, *V. tertius*, has a lower CV_A (~0.11) and an $s.d._A$ (~0.0015) that is similar to the $s.d._A$ of *V. aureus*. This raises the question of whether a relative measure (CV_A) or an absolute measure ($s.d._A$) is more appropriate for comparing intrinsic variation among species. For measures of body size (i.e. M), mean and variance are often positively related, suggesting the need for a relative measure of variation (Lewontin, 1966; Lande, 1977). However, it is not obvious that variation in allocation during development should scale with the amount of mass allocated to reproductive structures. Allocation is zero-sum, so allocating more to reproductive cells logically entails allocating less to somatic cells. Thus, if allocation variation increases with the level of allocation to reproduction, then it decreases with the level of allocation to soma. The dispersion–level relationship varies based on the seemingly arbitrary choice of focal category (reproduction or soma). The need for a relative measure of variation (and what that measure should be relative to) is not clear in the case of allocation (A). Therefore, we favour making between-species comparisons based on $s.d._A$ rather than CV_A. According to $s.d._A$, it appears that the two developmental-program-2 species, *V. africanus* and *V. carteri* f. *weismannia*, are substantially less precise at specifying cell-type numbers than *V. tertius*, *V. aureus*, and *V. rousseletii*. The unusual developmental feature shared by *V. africanus* and *V. carteri* f. *weismannia*, asymmetric divisions, is a derived character state likely absent from the ancestor of all *Volvox* (Herron *et al.*, 2010). If the high variation we observe results directly from this feature, then it, too, is a derived character state.

Particularly interesting is the difference in rate of division between the species with potentially high intrinsic variation (*V. africanus* and *V. carteri* f. *weismannia*) and those with potentially low intrinsic variation (*V. tertius*, *V. aureus*, and *V. rousseletii*). The latter group undergoes embryonic divisions much more slowly than the former group, consistent with a

speed–accuracy trade-off. Our data at this point are only suggestive, and the effects of colony size variation would need to be better controlled or accounted for to make strong conclusions concerning the overall level of absolute and relative variation intrinsic to each *Volvox* species.

With respect to differences between the observed and simulated data in the slope of the SMA line (right column, Fig. 4), the CV_A that produces data most similar to the observed data is less clear. In general, very low levels of CV_A appear to be ruled out for *V. africanus* and *V. carteri* f. *weismannia*, whereas the CV_A that matches the data best strongly depends on CV_M in the other three species. Clearly, the slope of the simulated data can change substantially depending on CV_A and CV_M. This is an important complication to keep in mind for studies that might aim to estimate the scaling exponent (to which M is raised in equation 2) from the slope of a regression on log-transformed gonidia and somatic cell data.

Our data suggest that *Volvox* species differ substantially in intrinsic variation of cell-type numbers. The level of dispersion created during development is an oft-neglected aspect of life history (Jacobs and Podolsky, 2010), and between-species differences in dispersion of cell-type number makes *Volvox* a promising system for future study of this topic.

Comparisons with other organisms

Newman and colleagues (2006) hypothesized that the early stages of metazoan multicellular life were characterized by high levels of variation in phenotypes arising from a single genotype; the subsequent evolution of developmental 'programs' led to reduced variability of phenotypes arising from a single genotype. If multicellular development in general follows this kind of scenario, then volvocine algal reproductive allocation is a promising trait to examine for evidence of this shift in variability.

Developmental systems often show a high degree of stability with respect to small (internal or external) perturbations, and this stability is thought to be an evolved property of developmental systems (e.g. Newman *et al.*, 2006). From a quantitative genetics perspective, the influence of development on the stability of a trait is captured in the special environmental effects. Special environmental effects capture variation within and among clones raised in the same macro-environment. Developmental stability has been addressed by studies of symmetry in bilaterally symmetric organisms; however, this approach only estimates one component of total special environmental effects (the within-individual component). Few studies have directly reported an estimate of the other (between-clone) component of special environmental effects (Lynch and Walsh, 1998, p. 113).

Assuming near genetic identity among individuals within each *Volvox* strain studied, our estimates of variance in gonidial cell number estimate the total special environmental effects and are comparable to other measures of variation in traits of clones raised in the same environment. The overall CV for fitness traits (e.g. litter size in pigs or egg number in poultry) among unrelated individuals is typically around 20% or more, and heritability is about 15% (Hill *et al.*, 2007). Thus, our estimates of among-clone variation in gonidial cell (i.e. offspring) number (Fig. 2) are higher than typical values of among-unrelated individual variation in fitness-related traits in domestic animals. The CV of clutch size in free-living striped plateau lizards was between 0.25 and 0.20 (Abell, 1999, calculated from table 1 therein, p. 175). For *Daphnia magna* asexual clones, the CV of clutch size for clones raised in the same environment was 0.17 for a high-food treatment and 0.32 for a low-food treatment (Guinnee *et al.*, 2004; T. Little, personal communication). The level of cell number (rather than offspring number)

variability observed in multicellular organisms is typically about a CV of 12% (Azevedo and Leroi, 2001), again lower than our *Volvox* observations.

In our conditions, the CV for offspring number in *Volvox* tended to be close to that observed for the harsher *Daphnia* conditions. Although the relatively low temperature in our experimental conditions is harsher than the conditions often used for culturing *Volvox*, these conditions were benign enough that populations of all species were growing rapidly at the time we sampled them, indicating that our high CV values are not an anomalous response to harsh conditions. Also, the lower number of offspring produced under these conditions may be more similar to offspring numbers observed in nature (Pentecost, 1983). *Pleodorina starrii*, a simpler relative of *Volvox*, has been observed also to have a high cell number CV (under constant external conditions), even higher than the *Volvox* CVs reported here (M. Herron, personal communication). This suggests that a connection between evolved developmental complexity and canalization of the phenotype may be borne out in the Volvocaceae.

Our data raise important questions about *Volvox* trait variability. Is high trait variability the ancestral character state of the first germ-soma differentiated species, with subsequent selection favouring developmental mechanisms that lower trait variability? Is the (potentially) high trait variability in *Volvox* compared with that of metazoans due to constraints imposed by its comparatively low morphological and developmental complexity? Alternatively, is high variability an adaptation in *Volvox* for particular ecological conditions?

Concluding remarks

Volvox colonies are striking in their physical manifestation of the two major fitness components: viability manifests in soma and fecundity manifests in gonidia. The simplicity of this body form, the developmental and ecological variety of the genus, and the direct connections to important evolutionary concepts such as fitness trade-offs make *Volvox* appealing for addressing the evolution of developmental mechanisms during the transition to full multicellular individuality.

This study provides fundamental data on the numbers of each cell type in asexual colonies of five *Volvox* species. We found among-species differences in the distributions and dispersion of gonidial cells per colony (Figs. 1 and 2). Although developmental mechanisms ultimately contribute to the level of dispersion in reproductive allocation for each species, we could not establish a correspondence (or lack thereof) between major developmental programs, as currently understood, and the overall level of dispersion in reproductive allocation. Variation in colony size was a significant confounding factor, as indicated by positive gonidia-soma correlations (Fig. 3). We attempt to account for this through a comparison of our data with simulated datasets using a mathematical model (Fig. 4). This analysis indicates that program-2 species (*V. africanus* and *V. carteri* f. *weismannia*) have substantially higher levels of intrinsic variability; however, further study is needed to confirm this result. The bivariate analysis also supported the comparatively low level of variability in *V. tertius* when relative variation (CV_A) is considered but not when absolute variation ($s.d._A$) is considered. For all species, the level of variability in offspring number was high compared with similar measures for more complex multicellular organisms, suggesting a connection between developmental complexity and canalization of the phenotype.

Finally, we observed mixed evidence for developmental constraints on cell numbers of *Volvox*. Only in *V. aureus* did somatic cells fall into two discrete categories, as has been described for *V carteri* f. *nagariensis*. For gonidia, *V. africanus* and *V. rousseletii* have distributions of gonidial number that are captured by a Poission distribution (Fig. 1), indicating that stochastic events are generating the observed variability in those cases. However, for the other species, the gonidial cell number distribution appears non-random, indicating that developmental bias is an important contributor to the observed distributions.

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