A Hydrodynamics Approach to the Evolution of Multicellularity: Flagellar Motility and Germ-Soma Differentiation in Volvocalean Green Algae

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abstract: During the unicellular-multicellular transition, there are opportunities and costs associated with larger size. We argue that germ-soma separation evolved to counteract the increasing costs and requirements of larger multicellular colonies. Volvocalean green algae are uniquely suited for studying this transition because they range from unicells to multicellular individuals with germ-soma separation. Because Volvocales need flagellar beating for movement and to avoid sinking, their motility is modeled and analyzed experimentally using standard hydrodynamics. We provide comparative hydrodynamic data of an algal lineage composed of organisms of different sizes and degrees of complexity. In agreement with and extending the insights of Koufopanou, we show that the increase in cell specialization as colony size increases can be explained in terms of increased motility requirements. First, as colony size increases, soma must evolve, the somatic-to-reproductive cell ratio increasing to keep colonies buoyant and motile. Second, increased germ-soma specialization in larger colonies increases motility capabilities because internalization of nonflagellated germ cells decreases colony drag. Third, our analysis yields a limiting maximum size of the volvocalean spheroid that agrees with the sizes of the largest species known. Finally, the different colony designs in Volvocales reflect the trade-offs between reproduction, colony size, and motility.

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Various selective pressures may push unicellular organisms to increase in size, but general constraints, such as the decrease in the surface-to-volume ratio, set an upper limit on cell size. Increase in size can also be achieved by aggregating mitotic products that are held together by a cohesive extracellular material, increasing the number of cells (instead of cell size). Natural selection has favored this strategy as illustrated by the multiple independent origins of colonial and multicellular organisms such as algae (Niklas 1994, 2000). Although large size has several benefits (e.g., predation avoidance, a buffered environment within a group), it is also associated with increased costs in terms of the time and energy required to produce a larger organism. Consequently, to maintain positive levels of fitness and allow for further increase in size, the benefits of larger size have to be increased and/or the costs have to be reduced. Here we argue that in volvocalean green algae, cell specialization evolved as a means to deal with the motility costs associated with increasing size.

Volvocales are aquatic flagellated organisms that comprise a monophyletic assemblage of lineages featuring varying degrees of complexity in terms of colony size, colony structure, and cell specialization. They range from the unicellular *Chlamydomonas* to colonies made of 4–64 cells with no cellular differentiation (e.g., *Gonium* and *Eudorina*) to multicellular individuals comprising 1,000–50,000 cells with complete germ-soma separation (e.g., *Volvox*; Koufopanou 1994; Kirk 1998; fig. 1). Specialization in reproductive and vegetative functions (i.e., germ-soma separation) characterizes the larger members of this lineage. From phylogenetic studies of the Volvocales, several inferences have repeatedly emerged (Coleman et al. 1994; Angeler et al. 1999; Coleman 1999; Fabry et al. 1999; Nozaki et al. 1999; Schagerl et al. 1999; Nozaki 2003). First,

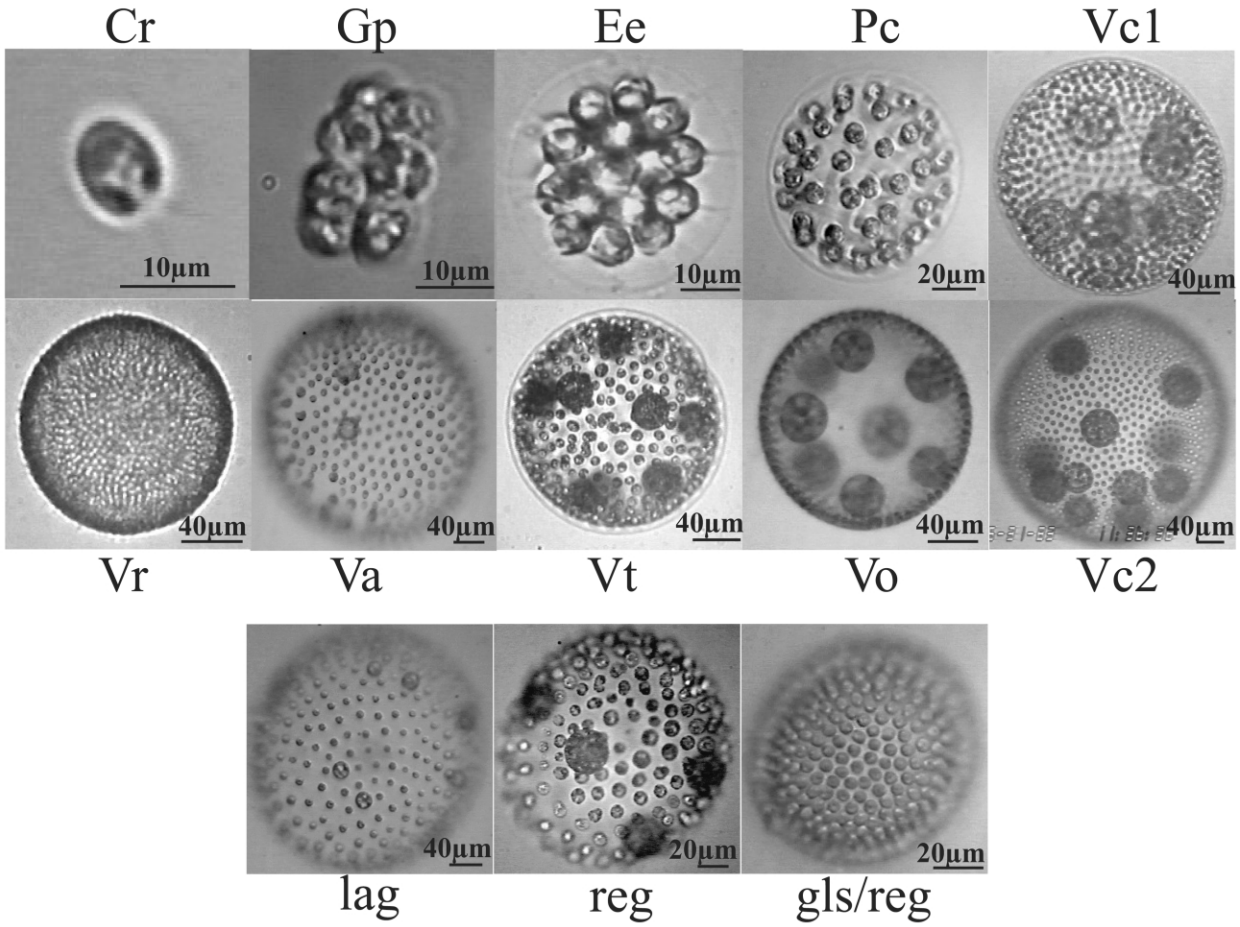

Figure 1: Subset of colonial volvocalean green algae and mutant forms derived from *Volvox carteri*, showing differences in cell number, volume of extracellular matrix, division of labor between somatic and reproductive cells, and developmental programs. Where two cell types can be identified, the smaller cells are the somatic cells, and the larger cells are the reproductive cells. The individuals in the images are representative of the synchronized populations that were used in the motility experiments. Images were captured when individuals just hatched. *Cr*, *Chlamydomonas reinhardtii* (UTEX 89); *Gp*, *Gonium pectorale* (UTEX LB 826); *Ee*, *Eudorina elegans* (UTEX 1201); *Pc*, *Pleodorina californica* (UTEX LB 809); *Vc1*, *V. carteri* grown at 600 foot-candles (fc; Eve strain; a subclone population separated from strain HK10, UTEX LB 1885); *Vc2*, *V. carteri* grown at 1,000 fc; *Vo*, *Volvox obversus* (UTEX LB 1865); *Vt*, *Volvox tertius* (UTEX LB 132); *Va*, *Volvox aureus* (UTEX LB 106); *Vr*, *Volvox rousseletii* (UTEX LB 1861); *lag*, *lag*$^{-}$ mutant (w153 k3 strain); *reg*, *regA*$^{-}$ mutant (153–68 strain); *gls/reg*, *gls/regA*$^{-}$ mutant (w238 strain). Eve and mutant strains were kindly provided by D. L. Kirk.

multicellular volvocalean algae have evolved from a common ancestor similar to the extant *Chlamydomonas reinhardtii* (Larson et al. 1992; Coleman 1999). Second, the germ-soma differentiated *Volvox* species have evolved several times independently from quite different ancestors (Coleman 1999; Nozaki et al. 1999; Nozaki 2003; fig. 2). Third, four developmental programs have also evolved several times independently (fig. 2; table 1; Desnitski 1995). Supporting this ease of evolutionary transition in Volvocales is the underlying genetic architecture responsible for the separation of germ and soma, which does not involve many genetic steps (Kirk 1997). For example, only two mutations are required to transform *Volvox carteri* into a mutant (*V. carteri glsA*$^{-}$/*regA*$^{-}$) with morphological and life-history features similar to those of *Eudorina* (Tam and Kirk 1991). In short, Volvocales comprise a group of closely related lineages with different degrees of cell specialization that seem to represent "alternative stable states" (Larson et al. 1992).

Volvocales are negatively buoyant (i.e., denser than water) and need flagellar beating to avoid sinking (Koufopanou 1994). They are found in quiet, standing waters of transient vernal puddles or in permanent lakes when thermal stirring stops and the lake becomes stratified (Reyn-

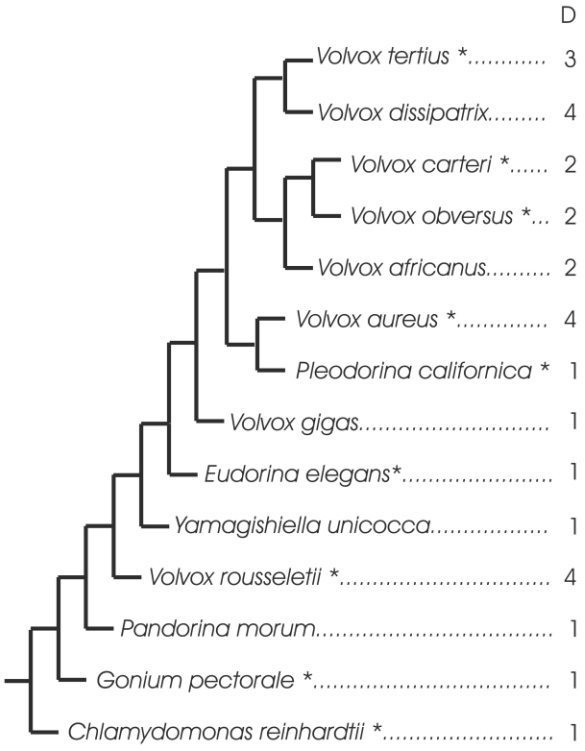

Figure 2: Phylogenetic relationships of Volvocales lineages modified from Nozaki (2003). Branch lengths do not indicate evolutionary distance. The first column is the developmental program number taken from Desnitski (1995). The species with asterisks were used in the experiments.

olds 1984; Kirk 1998). Hence, there is the belief that Volvocales need not only stay afloat but also have motility, which is crucial for the organism's viability because it allows it to control its position in the water column and to reach light and nutrients. In these still environments, higher motility capabilities probably give a competitive advantage over other nonmotile species. For example, Sommer and Gliwicz (1986) found that *Volvox* colonies migrated vertically several meters at night, presumably in search of higher phosphorous concentrations.

Koufopanou (1994) was the first to argue that in Volvocales, soma and the increase with size in the number of somatic cells (N_S) per reproductive cell (N_R; the N_S/N_R ratio) evolved to keep larger colonies afloat and motile while reproductive cells divide and develop. Undifferentiated volvocalean colonies, because of their rigid cell wall, are subject to the "flagellation constraint" because basal bodies cannot take the position expected for centrioles during cell division while still remaining attached to the flagella (as they do in naked green flagellates). Because a flagellum may beat for up to five cell divisions without the basal bodies attached, the 32-cell colony size (e.g., *Eudorina*) seems to be the critical threshold at which motility is severely compromised unless permanently flagellated cells evolve (soma). Koufopanou (1994) also showed that in Volvocales the N_S/N_R ratio increases with colony size and that the investment in somatic tissue increases twice as fast with size as does the investment in germ tissue; no direct evidence was given as to why a higher investment in somatic cells is needed for motility as colony size increases.

To test whether larger colonies need soma and whether the investment in soma needs to increase with size for colonies to stay afloat and motile, and to further clarify the hydrodynamic opportunities and costs of increased size and complexity, we study the swimming capabilities of various volvocalean species and mutant forms. A model based on standard hydrodynamics is used to describe the physical factors involved in motility as they relate to colony size, organization, and degree of germ-soma specialization. We then measure under controlled laboratory conditions the motility of the different colony types as well as the parameters and other variables used in the hydrodynamic model. We are the first to provide comparative measurements of swimming speeds and other fundamental hydrodynamic properties of an algal lineage composed of organisms of different sizes and degrees of complexity. The range of colony sizes enables the study of scaling laws: the number of cells N ranges from 10^0 to $\sim 10^4$. Finally, we discuss the trade-offs involved between investing in motility and reproduction and how they are manifest in the diversity of life forms in this lineage.

Hydrodynamic Model

We now develop a model based on standard hydrodynamics (Guyon et al. 2001) to elucidate the motility-related opportunities and physical constraints faced by colonies as they increase in size. Table 2 shows the notation used

Table 1: Developmental programs (*D1–D4*) of Volvocales as described by Desnitski (1995)

	D1	*D2*	*D3*	*D4*
Size of mature germ cells	Large	Large	Large	Small
Growth between divisions	No	No	No	Yes
Rate of divisions	Fast	Fast	Slow	Slow
Asymmetric division	No	Yes	No	No

Note: Developmental program 1 (*D1*) is considered the ancestral developmental program in this group (cell grows about 2^n-fold in size and then undergoes a synchronous series of n divisions). *D4* (e.g., *Volvox rousseletti*) is considered the most derived developmental program because palintomy is lost: reproductive cells start as small, flagellated cells, and during embryonic development, cells grow in between cell divisions (binary fission).

Table 2: Notation

Symbol	Definition
GS	Undifferentiated cells or colonies with GS cells only
G	Nonflagellated germ cells
S	Sterile flagellated somatic cells
GS/S	Colonies with GS and S cells
G/S	Colonies with G and S cells
GS/G	Colonies with GS and G cells
N	Total number of cells in a colony
N_R	Number of reproductive cells in a colony
N_S	Number of somatic cells in a colony
N_S/N_R	Somatic to reproductive cell ratio
D1–D4	Four developmental programs described by Desnitski (1995)
ECM	Extracellular matrix
Re	Reynolds number
g	Acceleration of gravity
η	Water viscosity
ρ_i	Density of i
$\Delta\rho_i$	Average difference in density between i and water
R	Colony radius
r, r_S	Average reproductive and somatic cell radius
r_F	Average flagellated cell radius
r_{max}	Maximum reproductive cell radius before the division phase
f	Average swimming force per flagellated cell
s	Proportion of somatic cells
q	Proportion of flagellated cells
$\bar{a}$	Weighted average of the flagellated cell area
$\bar{u}$	Weighted average of the cell volume
A	Intercellular surface area term
V_{sed}	Colony sedimentation speed
V_{up}	Colony upward swimming speed
ΔM	Difference in mass between the colony and the water displaced
n	Sample size

throughout the article. Because volvocalean algae colonies are small-diameter spheroids that swim at low velocities, they can be modeled as moving spheres in the low Reynolds number regime, $Re = RV\rho_w/\eta < 1$, where R is the colony's radius, V is the swimming or sedimentation speed, η is the viscosity of water, and ρ_w is the density of water. Even for a hypothetical large *Volvox* colony swimming at a considerable speed, $Re < 1$ (e.g., $Re = 0.25$ if $R = 0.05$ cm, $V = 0.05$ cm/s, $\eta = 10^{-2}$ g/s cm, $\rho_w = 1$ g/cm^3). Because the low Reynolds number regime applies to Volvocales, we can use the Stokes drag force acting on a moving sphere ($F = 6\pi\eta RV$). The effect of small deviations from sphericity is negligible (Happel and Brenner 1965).

At a low Reynolds number, a sedimenting sphere (i.e., colony) reaches a terminal velocity given by the equality between the Stokes drag force and the net force of gravity,

$$\Delta Mg = 6\pi\eta RV_{sed}, \tag{1}$$

where ΔM is the difference in mass between the colony and the water displaced, g is the acceleration of gravity, and V_{sed} is the sedimentation velocity. Note that $\Delta M = 4/3\pi R^3\Delta\rho$, where $\Delta\rho$ is the difference in density between the colony and water. Within this same framework, the force used by a colony to swim upward at a specific velocity (V_{up}) is the sum of the force overcoming drag and the force of gravity:

$$Nqf = 6\pi\eta RV_{up} + g\Delta M. \tag{2}$$

Solving for V_{up},

$$V_{up} = \frac{Nqf - g\Delta M}{6\pi\eta R}, \tag{3}$$

where q is the proportion of flagellated cells and f is the average effective upward swimming force per flagellated cell. The dependence of cell contribution on cell position is contained in the average, as are deviations from the Stokes drag due to flow near the sphere surface, generated

by the beating flagella. We return to these issues in the "Discussion."

We use the following notation to describe differentiation of reproductive and motility functions: GS refers to unspecialized flagellated cells performing both motility and reproductive functions successively (e.g., *Eudorina* cells and *Pleodorina* reproductive cells; fig. 1), G refers to nonflagellated cells specialized in germ functions (e.g., *V. carteri* and *Volvox obversus* reproductive cells; fig. 1), and S refers to sterile terminally differentiated flagellated cells specialized in somatic functions. In Volvocales, somatic cells do not divide after cleavage and stay small throughout colony development (e.g., *Volvox* species; fig. 1). In contrast, reproductive GS or G cells are similar in size to somatic cells when they are formed, but then they grow to produce the daughter colonies. For colonies containing more than one type of cell, a slash is used to separate the different cell types; for example, GS/S refers to a colony containing both unspecialized GS cells and somatic S cells.

In undifferentiated GS (e.g., *Eudorina*) and soma-differentiated GS/S (e.g., *Pleodorina*) colonies, $q = 1$ because all cells are flagellated. Because G cells are not flagellated, in germ-soma differentiated G/S colonies (e.g., *V. carteri*) $q = s$, where s is the proportion of S cells, N_S/N. We also assume that the flagellar beating force is the same for the two cell types, GS and S. Thus, V_{up} depends on the total swimming force that a colony is able to generate (Nqf) minus its gravitational force ($g\Delta M$) divided by a drag factor ($6\pi\eta R$) that depends on the colony radius. Note that the drag factor can only decrease the absolute value of V_{up}, but the gravitational force can turn V_{up} negative, making the colony sink. The three terms depend on the size of the colony (N), on its organization (e.g., G/S), and on the proportion of flagellated (q), reproductive ($1 - s$), and somatic (s) cells. We can then use equation (3) as a proxy for the motility capability of colonies of different sizes and degrees of cell specialization.

We developed a simple geometric model to calculate the mass (i.e., ΔM) and radius (R) of the different colony types and to understand colony organization. Three cell types are considered: GS, G, and S (see app. A for model details). The colony ΔM is composed of the difference in mass between the cells and the water they displace and the difference in mass between the extracellular matrix (ECM) and the water it displaces (app. A, eq. [A1]). We assume in the model that colonies and cells are spheres, that the density of the different cell types is the same, and, because of the apparently aqueous nature of the ECM, that the difference in density between the ECM and water is negligible ($\rho_{ECM} = \rho_w$; app. A, eq. [A2]). Colony radius R depends on the number of flagellated cells Nq (GS and/or S cells) and on the area between cells (app. A, eq. [A3]). We model flagellated cells as circles arrayed on the sphere surface. For simplicity, the intercellular space term A is not taken into account ($A = 0$; app. A, eq. [A4]). By using equations (A2) and (A4) for ΔM and R, respectively, equation (3) expanded becomes

$$V_{up} \approx \left(\frac{qf - g(4/3)\pi[(1 - s)r_{max}^3 + sr_S^3]\Delta\rho_C}{3\pi\eta\{[1 - (s/q)]r_{max}^2 + (s/q)r_S^2\}^{1/2} q^{1/2}} \right) N^{1/2}, \quad (4)$$

where r_S is the radius of S cells, $\Delta\rho_C$ is the difference in density between the cells and water, and s/q is the proportion of S cells from the total number of flagellated cells, N_S/Nq. Because we assume that colonies need to keep afloat and motile while reproductive cells divide and develop, to analyze the model we use the size r_{max} that the reproductive cell (GS or G) has to reach to produce a colony of the same type. The larger the colony, the larger the size the reproductive cell has to reach to produce a colony of the same type. Thus, r_{max} is a function of the number of cells (N), the initial cell size (r_{in} and r_{Sin} for reproductive and somatic cells, respectively), and the proportion of cell types in that colony (app. A, eq. [A6]).

Perhaps due to phylogenetic constraints, strategies for avoiding sinking and yet maintaining motility (e.g., increasing the number of flagella per cell or developing gas vacuoles for buoyancy regulation as in *Coelospaherium*) did not appear in the Volvocales (Graham and Wilcox 2000). Thus, we begin by analyzing the simplest case to find out whether a colony composed of *C. reinhardtii*–type cells needs to invest in somatic cells and whether the proportion of somatic cells (s) needs to increase as colony size increases to keep the colony afloat (i.e., $V_{up} = 0$). For simplicity, in all the analyses, the flagellar beating force f and the cells' density are fixed to *C. reinhardtii* values (fig. 3). The initial cell sizes after cleavage and the size of somatic cells are fixed to the same value because somatic cell sizes are small and similar in the different species ($r_{in} = r_{Sin} = r_S$; app. A, eq. [A6]). By assuming $V_{up} = 0$, the drag term ($6\pi\eta R$) disappears and the flagellar beating force only needs to counteract the downward gravitational force ($Nqf = g\Delta M$). Equation (4) becomes

$$qf = g\frac{4}{3}[(1 - s)r_{max}^3 + sr_S^3]\pi\Delta\rho_C. \quad (5)$$

Solving equation (5) for the proportion s of somatic cells for GS/S colonies ($q = 1$),

$$s = -\frac{3f - g4\pi r_{max}^3\Delta\rho_C}{g4\pi(r_{max}^3 + r_S^3)\Delta\rho_C}, \quad (6a)$$

and for G/S colonies ($q = s$),

$$s = \frac{g4\pi r_{\max}^3 \Delta\rho_C}{3f + g4\pi(r_{\max}^3 - r_S^3)\Delta\rho_C}. \quad (6b)$$

Figure 3 shows how s changes as a function of N when $Nqf = g\Delta M$ (eqq. [6]) so as to maintain the colonies' buoyancies. For colonies to avoid sinking as they increase in size, the model predicts that they must invest in a higher proportion s of somatic cells, thereby increasing the N_S/N_R ratio. The flagellar beating force (Nqf) has to increase to compensate for the increase in the downward gravitational force ($g\Delta M$) caused by the increase in size of the reproductive cells needed to produce larger colonies (r_{max}, app. A, eq. [A6]). If we compare GS/S to G/S colonies, GS/S colonies have the benefit of all their cells being flagellated, increasing the total colony beating force Nqf. The G/S colonies have the cost of G cells not contributing to Nqf. Note that for smaller colonies, GS/S colonies need a smaller proportion of somatic cells to stay afloat than do G/S colonies. But, as size increases, the difference between the two colony types becomes negligible because the proportion $(1 - s)$ of reproductive cells becomes very small; thus, the contribution of GS cells to the total flagellar beating force to keep the colony afloat is also very small.

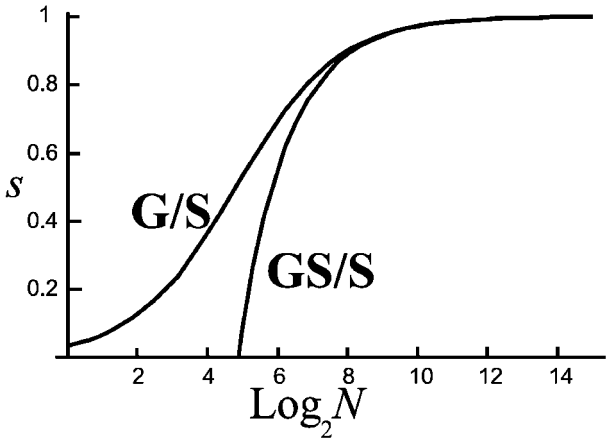

Figure 3: Proportion s of somatic cells needed by colonies of *Chlamydomonas reinhardtii*–type cells to avoid sinking. Equations (6) (for colonies with unspecialized reproductive cells and soma [GS/S] and for colonies with specialized germ and soma [G/S], respectively) are plotted as a function of number of cell divisions ($\log_2 N$). When $s = 0$, colonies only have undifferentiated GS cells. The parameters used were taken from measurements performed on newly hatched *C. reinhardtii*: $\Delta\rho_C = 0.047$ g/cm^3 (see table C1 in the online edition of the *American Naturalist*), $r_{in} = r_{Sin} = r_S = 0.00035$ cm (see table B1 in the online edition of the *American Naturalist*), and V_{up}, r, and $\Delta\rho_C$ were used to calculate f (2.4×10^{-7} dyn; table B1). For colonies to avoid sinking as they increase in size, they must invest in a higher proportion s of somatic cells. Note that for smaller colonies, GS/S colonies need a smaller proportion of somatic cells than G/S colonies to stay afloat because GS cells are flagellated. As size increases, the difference between the two colony types becomes negligible because the proportion $(1 - s)$ of reproductive cells becomes very small.

Because motility (self-propulsion) is important for colony viability (explained above), we now analyze the model with the drag term ($6\pi\eta R$) included to calculate the colony's swimming speed as colonies increase in size (eq. [4]). Figure 4*A* shows how V_{up} changes as a function of colony size for different s in GS/S colonies (all cells perform motility and are on the surface). Note how, as the number of cells (N) increases for a fixed s, first the swimming speeds increase because the swimming force (Nqf) increases more than the downward gravitational force ($g\Delta M$; fig. 4*B*) and the drag (R). But as size continues to increase, this trend reverses, and the swimming speeds abruptly decline and reach negative values. This happens because there is a larger increase in the downward gravitational force (ΔM) and the drag (R) compared to the increase in swimming force due to the increase in size of the reproductive cells (r_{max}; app. A, eq. [A6]). Figures 3 and 4*A* show that even in the absence of the "flagellation constraint" (discussed above; Koufopanou 1994), undifferentiated GS colonies ($s = 0$) reach a threshold size at which they sink unless they invest in somatic cells.

If we compare GS/S to G/S colonies for swimming speeds, GS/S colonies have the benefit of all their cells being flagellated, increasing the swimming force Nqf, but G/S colonies have the benefit of the unflagellated G cells growing in the interior of the colony, thereby decreasing the colony surface area and drag. When s is small, GS/S colonies have higher motility capabilities than G/S colonies, but as s increases, the situation reverts, and G/S colonies have higher motility (fig. 4*C*). Consequently, in large colonies with the proportion of somatic cells s close to 1, the model predicts that germ specialization benefits motility because the benefit of decreased drag outweighs the cost of decreased total colony swimming force Nqf. Now, let us compare the same type of colony (e.g., G/S) with different developmental modes: a G/S colony in which initially germ cells are small (e.g., *D1* colonies) and a G/S colony in which initially germ cells are large (e.g., asymmetric division, *D2* colonies). Figure 4*D* shows that because of the increase in gravitational force $g\Delta M$ in colonies with larger germ cells, not only does V_{up} decrease but also the upper size limit at which colonies sink decreases significantly.

The results of the hydrodynamic model show that as colony size increases, soma specialized in flagellar motility must evolve, the somatic-to-reproductive cell ratio increasing to keep colonies buoyant and motile while reproductive cells divide and develop. Increasing investment in reproductive tissue (by increasing the size and/or number of reproductive cells) decreases motility and vice versa. These results are based on several assumptions, some con-

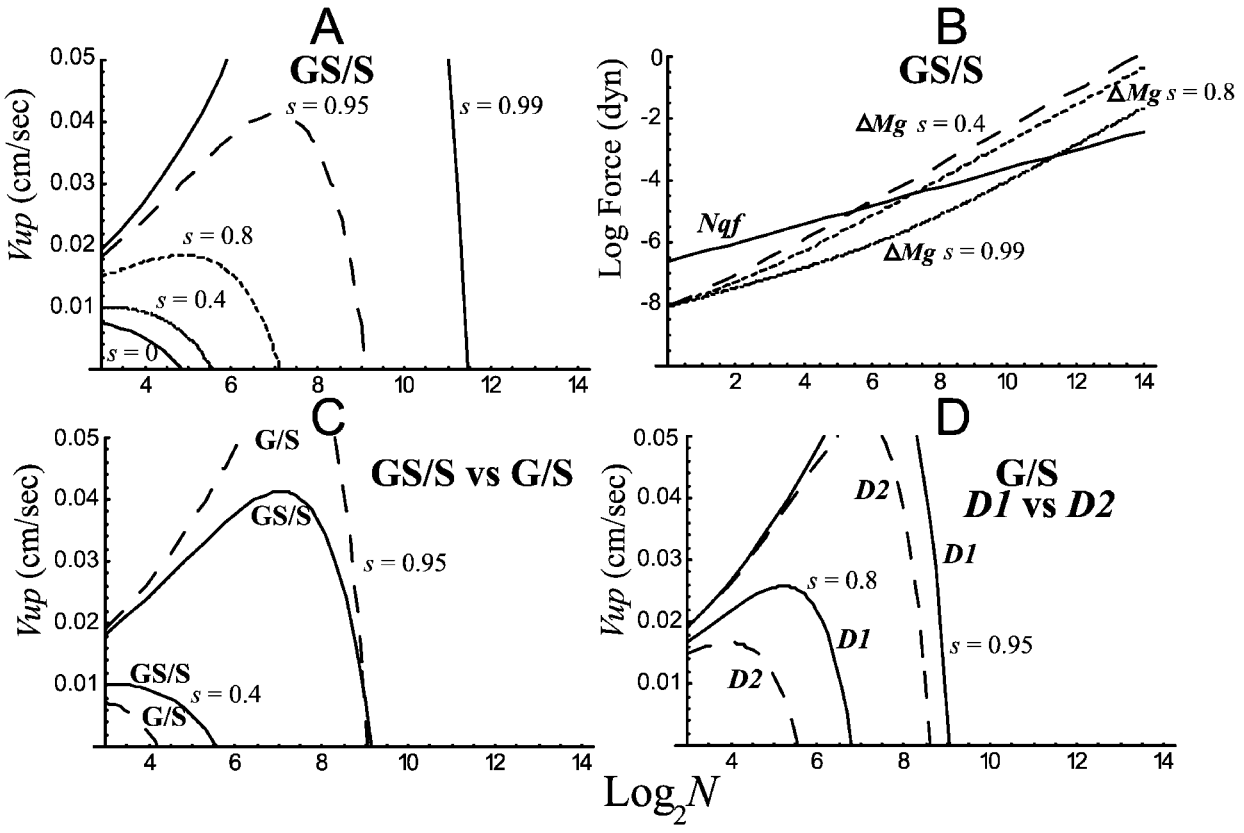

Figure 4: Constraint of size on swimming speeds. V_{up} (cm/s; eq. [4]) as a function of number of cell divisions ($\log_2 N$) for different proportions s of somatic cells. The same values in figure 3 are used for $\Delta\rho_C$, r_{in}, r_{Sin}, r_S, and f. *A*, V_{up} of GS/S colonies for different s ($s = 0$ is for GS colonies). As colony size (N) increases for a fixed s, first the swimming speeds increase because the swimming force (Nqf) increases more than the downward gravitational force ($g\Delta M$; *B*). But as size continues to increase, the swimming speeds abruptly decline and reach negative values due to the increase in size of the reproductive cells that need to produce larger colonies (r_{max}; app. A). *B*, Nqf and ΔMg of GS/S colonies for different s. This figure shows how Nqf and $g\Delta M$ change and cross as colony size increases for a fixed s. At the threshold size, when $Nqf < g\Delta M$, colonies sink. *C*, GS/S versus G/S colonies with same s. When s is small, GS/S colonies have higher swimming capabilities because of the benefit of all their cells being flagellated. As s increases, the situation reverts, and G/S colonies do better because the benefit of decreased drag outweighs the benefit of increased flagellar force. *D*, *D1* versus *D2* colonies with same s. To simulate asymmetric division for *D2* colonies, $r_{in} = 2r_{Sin}$. Because of the increase in gravitational force $g\Delta M$, in colonies with larger initial germ cell size, not only V_{up} but also the upper size limit at which colonies sink decrease.

servative, others not. If the difference in density between the ECM and water is not negligible, the need to invest in somatic cells would be even higher because ΔM would increase. Species differ in their cell-surface concentration pattern. Not using the intercellular space term A when analyzing the model might lead to an understatement of R, therefore also leading to an understatement of the need of somatic cells for motility. For example, *Volvox gigas* reaches a diameter of 3 mm or more but usually contains <2,000 cells (Van de Berg and Starr 1971). Besides, the assumption of a fixed f, r_S, and $\Delta\rho_C$ needs to be confirmed for real colonies. The average force per flagellated cell f, reproductive or somatic, might be dependent on colony and cell size. As a result of constructive or destructive interference, the change in Nq per colony and per unit area may increase or decrease the force efficiency of each cell. Also, the sizes of flagellated cells (r or r_S) vary between species, and flagellated cells grow as the colony develops. We do not yet know whether there is any relationship between cell size and flagellar beating force. Cell density $\Delta\rho_C$ may also vary between cell types and species, changing ΔM.

To investigate the assumptions used when analyzing the model as well as the results reached, we measured the parameters used in the equations and analyzed them as a function of size (N). We measured the swimming (V_{up}) and sedimentation (V_{sed}) velocities, cell (r and r_S) and colony (R) size, and number and proportion of cells (N, Nq, s) of various volvocalean algae species of different sizes. The measurements were made on synchronized popula-

tions of newly hatched colonies and at time intervals as colonies developed. From these measurements we calculated the total force Nqf, the force per flagellated cell f, and the difference in mass between the colony and the displaced water ΔM (eqq. [1], [2]). We expect the size-dependant allometric analysis of these measurements to inform us about possible important associated parameter changes (i.e., f, $\Delta\rho_C$, cell size) in relation to colony size (N) and to confirm (or not) the results yielded by the model analysis. We also compare the motility capabilities of the different developmental forms and mutants to investigate the trade-offs between investing in motility, reproduction, and size.

Methods

Synchronous cultures of asexual colonies were grown in standard *Volvox* medium (SVM), cool white light (~1,000 foot-candles [fc]), and 16L/28°C : 8D/26°C cycle (Kirk and Kirk 1983). The species used in the experiments were chosen to represent the range of sizes, developmental modes, and degrees of cell specialization observed in Volvocales (fig. 1). The three mutants derived from *Volvox carteri* used show alternative colony designs that can help elucidate the effects that cell specialization, developmental mode, and colony organization have on motility in this group. Table 3 shows all the data collected for all the species/mutants used in this study.

To measure cell size, the orthogonal diameters of two randomly chosen reproductive cells and five somatic cells were measured (averages were used). To count the number of cells in the larger colonies, we used a sample area from the two sides of the sphere (i.e., the anterior side and the posterior side; Kirk 1998). The cell count in those areas was averaged to calculate the total number of somatic cells (using the averaged measured colony diameter and assuming colonies are spheres).

All species and mutants swam upward when placed in the dark (gravitaxis due to anisotropic distribution of internal mass; Kessler 1986). Thus, upward swimming velocities were recorded (cm/s, V_{up}) in the dark using a light with an infrared filter. Algae did not detect the infrared wavelength because they only swam toward the light on the side when the filter was absent. After colonies hatched, stock populations were randomly sampled for measurements every 2 h. From each measurement, 15 individuals were sampled to measure number and size of cells and colony size.

Colonies were placed in an air sealed 4-cm^3 glass cuvette in a 28°C water bath to control for convection currents and gas gradients. Once the colonies reached the top of the cuvette, the cuvette was inverted to allow for colonies

Table 3: Description and data measured for the colonies used in the experiments

Species	CT	D	GT	HT	n	Mean N_R	SE	Median N_R	Mode N_R	Mean N_S	SE	N	N_S/N_R	SE
Chlamydomonas reinhardtii	GS	1	1	0	…	1	…	1	1	0	…	1	0	…
Gonium pectorale	GS	1	1	0	30	9.3	.6	8	8	0	…	9	0	…
Eudorina elegans	GS	1	1	0	30	20.5	1	17	16	0	…	21	0	…
Pleodorina californica	GS/S	1	3	5–6	10	54.8	6	52.4	…	40.3	3.9	95	.77	.07
Volvox obversus 600 fc	G/S	2	3	3–4	30	8.3	.2	8	8	883	48	891	106	6
Volvox carteri 600 fc	G/S	2	2	1–2	20	8.7	.3	8	8	1209	69	1218	140	7
V. carteri	G/S	2	2	1–2	20	12	.4	12	11	2190	93	2202	185	11
V. carteri regA$^-$	G/GS	2/1	…	…	10	3.9	.5	4	4	239	59	243	60	14
V. carteri gls/regA$^-$	GS	1	3	3–4	30	561	48	544.5	…	0	…	561	0	…
V. carteri lag$^-$	GS/S	1	2.2	1–2	20	9.5	.5	9	9	856	111	866	91	13
Volvox tertius	G/S	3	2.5	7–8/1–2	30	12.8	.5	13	13	1125	38	1138	88	3
Volvox aureus 600 fc	G/S	4	3	3–4	30	5.1	.2	5	5	1630	101	1635	338	27
Volvox rousseletii	G/S	4	2	7–8	20	12.6	1.3	11	10	3065	343	3078	243	20

Note: Colonies grown under the conditions described in the "Methods" section. CT = colony type. D = developmental mode as described by Desnitski (1995). GT = consistent generation time when synchronized in the conditions outlined; ~80% of *lag*$^-$ colonies hatched in 2 d and ~20% in 3 d; when *Volvox tertius* colonies hatched 2 h into the light cycle, those colonies would hatch 2 d later, 8 h into the light cycle, and vice versa. HT = hatching time; number of light hours before hatching. n = sample size. *Volvox carteri* was synchronized both at ~600 and ~1000 foot-candles (fc), and *Volvox aureus* and *Volvox obversus* only at ~600 fc because these strains either grew deficiently or bleached and died at ~1000 fc. *Volvox obversus* and *Volvox rousseletii* are male strains. The *V. rousseletii* strain used for the experiments had a low proportion of spontaneous sexual colonies (with sperm packets instead of gonidia). The *lag*$^-$ mutant germ cells start as small undifferentiated GS cells and perform motility functions before reproducing (GS/S; Kirk 1988). These mutants have a higher proportion of extracellular matrix, forming larger spheroids than the wild types. When they hatch, *regA*$^-$ mutants (GS/G; somatic cells regenerate to become reproductive; Starr 1970; Huskey and Griffin 1979) resemble wild-type colonies, but in the end all cells contribute to offspring. Colonies are much smaller than wild type because most of the progeny come from regenerated somatic cells. The *gls/regA*$^-$ mutants form large undifferentiated GS colonies, larger than any undifferentiated extant species known (Tam and Kirk 1991).

to swim up again and for swimming trajectories to be recorded (V_{up}). After V_{up} was recorded, individuals from the same population were deflagellated to record sedimentation velocity (V_{sed}). Deflagellation was achieved by lowering the pH with a mild acid for 30 s. When colonies were deflagellated, no osmotic change was noted. Colonies did not change in size and would regenerate their flagella and swim after ~30 min; cell density also did not change (measured in *V. carteri* using continuous Percoll gradient; see app. C in the online edition of the *American Naturalist*). Individuals were placed in the same setup and the cuvette was inverted to record sinking trajectories (V_{sed}). Currents always settled before recording started. An optical bench was used for videotaping. V_{up}, V_{sed}, and direction of the trajectories were then calculated using Motion Analysis software (ExpertVision 2D/AT, version 3.1.; Motion Analysis 1990). Trajectory durations captured ranged from 1 to 5 s. Net velocities (controlling for tortuosity of the trajectory) were used for the analysis. When a proportion or all of the colonies were not swimming, V_{up} measurements were not recorded.

For the size-dependent (allometric) analysis, simple (SLR) and multiple (MLR) additive linear regression was used. To check for phylogenetic constraints, we also used the independent phylogenetic contrast method on R, V_{sed}, and V_{up} (Compare 4.6 software package; Felsenstein 1985). The phylogeny in figure 2 was used, and branch lengths were set to 1 (the branch lengths accuracy does not seem to have a large effect on the independent contrast results; Martins and Garland 1991). When P values are not reported, $P < .0001$.

Quantification of the Model

Overview

Table B1 in the online edition of the *American Naturalist* shows the entire data set: the average V_{up}, V_{sed}, R, r, and r_S measured for all the species and mutant forms for the time series after hatching. Figure 5 shows some general trends of the data given in table B1. Note that just after hatching, the colonies of larger species (e.g., *Volvox*) tend to have higher swimming speeds than the colonies of smaller species (e.g., *Chlamydomonas* and *Gonium*); as species develop and their components enlarge, their swimming speeds decrease and their mass and radius increase. *Volvox rousseletii* is the fastest swimmer; it swims 10 times faster than *Chlamydomonas reinhardtii*.

We have organized our analyses of these data in four parts. First, we perform a size-dependant allometric analysis on the measured and calculated parameters of newly hatched extant colonies as a function of N (section A). Second, we analyze the swimming force and speed as colonies develop (section B). Third, we insert into the model the measured and calculated parameters so as to calculate the physical limits of design in this lineage (section C). Finally, we compare differences in the swimming force and speed between developmental programs and mutant forms (section D).

Allometric Analysis as a Function of N *of Newly Hatched Extant Colonies*

In this section we test for the more general size-dependent relationships predicted by the model to see whether the model is robust over the four orders of magnitude of size spanned by the Volvocales (N from 10^0 to ~10^4). The model predicts that colony radius should be proportional to colony size to the one-half power, $R \propto N^{1/2}$, if, as assumed, the weighted average of the flagellated cell area ($\bar{a} = \{1 - [s/q]\}r^2 + [s/q]r_S^2$) and the intercellular space area (A) do not change significantly as a function of N (app. A, eq. [A4]). When plotting log R versus log N, SLR yields $R \propto N^{0.47}$, not significantly different from 0.5 (fig. 6). This shows that when comparing the newly hatched colonies of extant species, the flagellated cell size $\bar{a}$ and the intercellular space A do not seem to vary significantly in relation to N. The SLR yields that $\bar{a}$ is invariant in relation to N ($P = .23$).

The model also predicts that the difference in mass between the colony and the displaced water should be proportional to size, $\Delta M \propto N$, if, as assumed, the weighted average of the cell volume ($\bar{u} = [1 - s]r^3 + sr_S^3$) and the difference in density between the cells and water ($\Delta\rho_C$) do not change significantly as a function of N (app. A, eq. [A2]). If the measured R and V_{sed} values of the extant species are inserted in equation (1) to calculate ΔM, SLR yields $\Delta M \propto N^{1.08}$, not significantly different from 1 (fig. 6). Nevertheless, the exponent is >1 partly because cell volume $\bar{u}$ increases as N increases ($\bar{u} \propto N^{0.12}$, SE = 0.05, $r^2 = 0.27$, $P = .02$). To analyze $\Delta\rho_C$, we measured the cell densities of three species (app. C). We found that the densities of *C. reinhardtii* and *Gonium pectorale* cells and of *Volvox carteri* somatic cells do not differ significantly (~1.05 g/cm^3), but the density of large germ cells in newly hatched *V. carteri* colonies is significantly lower than that of the other cell types (~1.02 g/cm^3). In conclusion, evidence shows that the increase in cell volume, $\bar{u}$, as N increases is a consequence of some of the newly hatched *Volvox* colonies having large reproductive cells (e.g., *V. carteri*; table B1). Also, the average cell density $\Delta\rho_C$ of colonies having large reproductive cells might decrease because large germ cells have a lower cell density.

When plotting the log of the upward swimming speed V_{up} versus log N, SLR yields $V_{up} \propto N^{0.27}$ (fig. 6), significantly lower than the 0.5 exponent expected if deviations

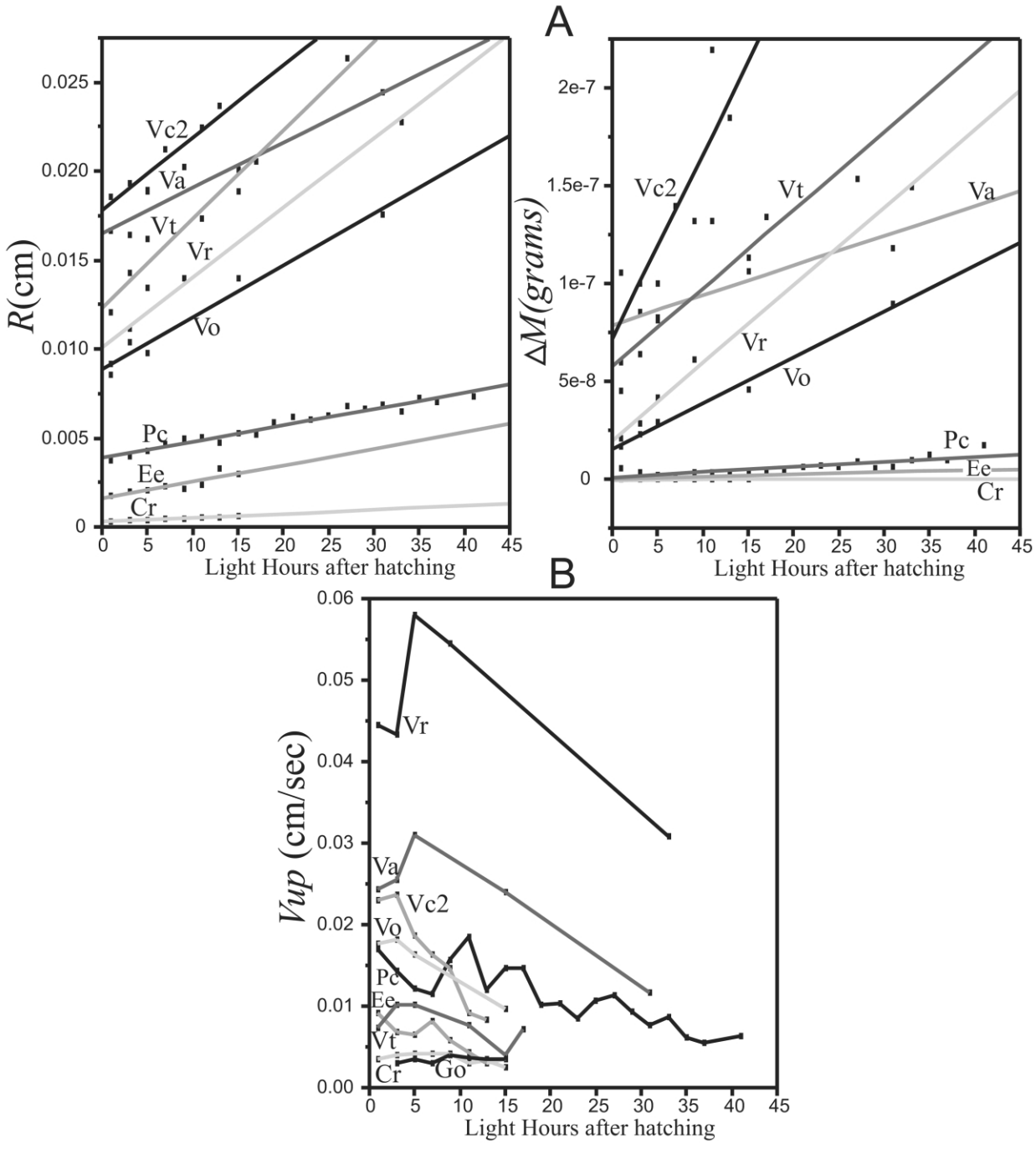

Figure 5: Change in size and swimming speeds as colonies develop. *A*, *R* and ΔM as a function of time for the wild types. Simple linear regression (SLR) is used to represent dR/dt and $d\Delta M/dt$ for each species. All the slopes of the SLR have $P < .05$. *B*, V_{up} as a function of time for the wild types. Time-series points are joined for each species. *Cr*, *Chlamydomonas reinhardtii*; *Gp*, *Gonium pectorale*; *Ee*, *Eudorina elegans*; *Pc*, *Pleodorina californica*; *Vc2*, *Volvox carteri* grown at 1,000 foot-candles; *Vo*, *Volvox obversus*; *Vt*, *Volvox tertius*; *Va*, *Volvox aureus*; *Vr*, *Volvox rousseletii*. *Volvox carteri*, *V. obversus*, and *V. tertius* were not able to swim once the daughter colonies formed inside.

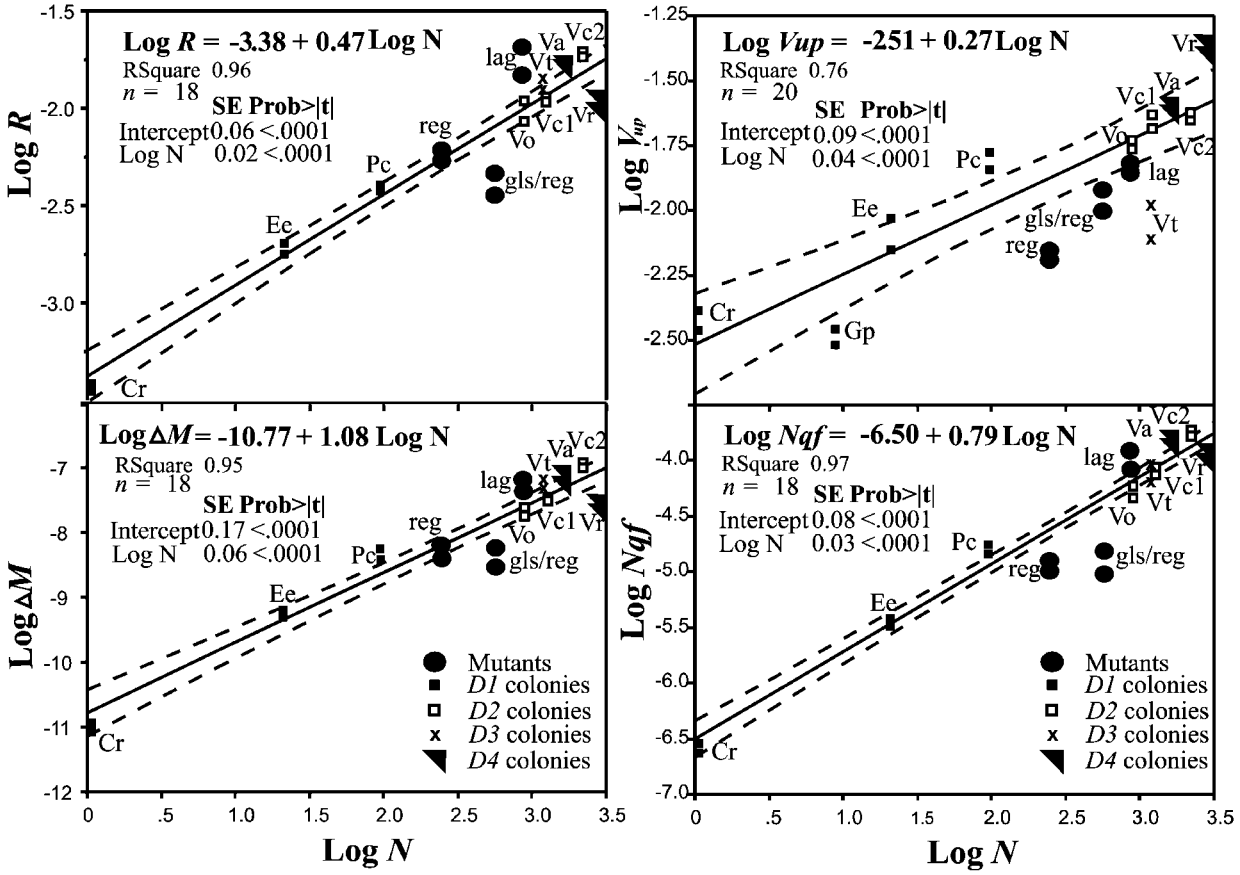

Figure 6: Allometric analysis. *R*, ΔM, V_{up}, and *Nqf* as a function of *N*. Mutants are shown but not used in the analysis. Equation (1) was used to calculate ΔM (g) from V_{sed} (cm/s) and *R* (cm; $\eta = 10^{-2}$ g/s/cm; $g = 980$ cm/s^2). In equation (2), V_{up} (cm/s), V_{sed}, and *R* were used to calculate the total colony force *Nqf* (dyn). Only the first two measurements of the time series were used for the interspecies allometric analysis of newly hatched colonies (1 and 3 h after algae having hatched; table B1). For all the relations analyzed as a function of *N*, the measurements made 1 and 3 h after algae hatching did not show any significant slope or intercept difference. *Cr*, *Chlamydomonas reinhardtii*; *Gp*, *Gonium pectorale*; *Ee*, *Eudorina elegans*; *Pc*, *Pleodorina californica*; *Vc1*, *Volvox carteri* grown at 600 foot-candles (fc); *Vc2*, *V. carteri* grown at 1,000 fc; *Vo*, *Volvox obversus*; *Vt*, *Volvox tertius*; *Va*, *Volvox aureus*; *Vr*, *Volvox rousseletii*; *lag*, *lag*$^-$ mutant; *reg*, *regA*$^-$ mutant; *gls/reg*, *gls/regA*$^-$ mutant.

are not found due to associated parameters (*f*, $\Delta\rho_C$, $\bar{a}$, and $\bar{u}$; eq. [4]). When correcting V_{up} for cell size with the measured $\bar{a}$ and $\bar{u}$, the exponent does not change significantly, still remaining lower than 0.5. Also, an increase in $\Delta\rho_C$ as *N* increases would negatively affect the V_{up} exponent, although we found no evidence for this inference. Thus, the average swimming force per flagellated cell *f* must decrease as a function of *N*, lowering the exponent of the relation between V_{up} and *N*. If the measured *R*, V_{sed}, and V_{up} values of the extant species are inserted in equation (2) to calculate the total swimming force *Nqf*, $Nqf \propto N^{0.79}$ when SLR is used (fig. 6). Because $Nqf \propto N^{0.79}$, the swimming force per cell $f \propto N^{-0.21}$ given that the proportion of flagellated cells *q* is essentially invariant ($q = 1$ in GS or GS/S colonies, and $q > 0.98$ in the G/S colonies measured). In conclusion, as the number of cells *N* increases, the average contribution made by flagellated cells to the total swimming force of the colony decreases, decreasing the exponent of the relation between V_{up} and *N*.

In summary, when comparing the analysis of the model

Table 4: Variation of parameters as powers (β) of *N*

	R	V_{sed}	V_{up}	ΔM	$\bar{u}$	*f*
β	.47 (.5)	.6 (.5)	.27 (.5)	1.08 (1)	.12	−.21
β_I	.43	.51	.28	…	…	…

Note: Numbers in parentheses are the theoretical expected values of these exponents if the associated parameters (e.g., $\bar{a}$, $\Delta\rho$C; app. A) do not change in relation to colony size (*N*). β_I powers are taken from the independent phylogenetic contrast analysis (*R*, SE = 0.04; V_{sed}, SE = 0.07; V_{up}, SE = 0.08).

(where we fixed the values of the flagellar beating force f, the cell density $\Delta\rho_C$, and the size of somatic cells r_S) to the experimental data, the main relationship found with colony size N that significantly affects the results of the model is a decrease in f as N increases. Table 4 reports the exponents of the parameters measured and calculated as a function of N. When plotting log R, V_{sed}, and V_{up} versus log N using the independent phylogenetic contrast method, the exponents do not significantly differ from those yielded by SLR.

Analysis of Colonies as They Develop

In this section we focus on changes in size during the development of each species. These results are especially informative because they are for a single organism as it increases in size during development. They specifically let us analyze how the force per flagellated cell f changes as cell size increases.

When colonies develop, because the proportion s of soma and the number of cells N remains fixed, the colony radius R increases due to an increase in cell size (flagellated cell area $\bar{a}$) and accumulation of extracellular matrix ECM (intercellular space area A). Assuming cell density does not decrease (app. C), the difference in mass between the colony and the displaced water ΔM increases due to the increase in cell mass caused by the enlargement of cells (cell volume $\bar{u}$) as the colony develops (mainly due to the reproductive cells or embryos). In contrast, figure 5*B* shows how the upward swimming speed V_{up} tends to decrease as colony size increases.

When analyzing log V_{up} versus log $N + \log \bar{u}$, MLR yields $V_{up} \propto N^{0.27}\bar{u}^{-0.32}$ (N and $\bar{u}$ exponent SE = 0.02 and 0.07, respectively; $n = 68$, $r^2 = 0.75$). This means that after correcting for the increase in V_{up} due to N, on average V_{up} decreases as the average cell volume of the developing colonies increases. On the other hand, when analyzing the log of the average force per flagellated cell f versus log $N + \log \bar{a}$, MLR yields $f \propto N^{-0.21}\bar{a}^{0.51}$ (N and $\bar{a}$ exponent SE = 0.01 and 0.6, respectively; $n = 61$, $r^2 = 0.91$). This means that after correcting for the decrease in f due to N, f roughly increases linearly to the average radius of flagellated cells ($f \propto r_F^{1.02}$). In conclusion, as cells increase in size, the increase in the average force per flagellated cell ($f \propto r_F$) is lower than the increase in cell volume ($\bar{u} \propto r^3$). Thus, the colony swimming speed (V_{up}) decreases as colonies develop because the increase in f is not sufficient to compensate the increase in cell mass (i.e., ΔM), roughly $f \propto \bar{u}^{1/3}$. Complementary analysis shows that the flagella of *V. carteri* somatic cells increase significantly in length as these cells increase in size, but the flagellar beating rates have a small decline (app. C). Thus, we speculate that larger cells have higher flagellar beating force due to increasing flagellar length, but further studies are needed.

Interestingly, not only did the V_{up} of *lag*$^-$, *D2*, and *D3* colonies decrease as these colonies developed, but also these colonies were not able to swim once their daughter colonies formed inside. The hypothetical V_{up} of these colonies when they do not swim was calculated (app. C). These values were negative, thereby confirming that these colonies sink because their ΔM is too high (table B1). The *gls/regA*$^-$ undifferentiated GS colonies stopped swimming when the somatic cells regenerated and reabsorbed their flagella, but these colonies would sink anyway because their hypothetical V_{up} values become negative. In contrast, *D4* colonies were able to swim until the daughter colonies hatched (*Volvox aureus* and *Volvox rousseletii*; fig. 5).

How can daughter colonies survive in nature if they spend one day inside their mother colonies deprived of swimming capabilities (e.g., *V. carteri*)? We placed *V. carteri* synchronized colonies with fully formed daughter colonies inside in the dark for 5 h, and most daughter colonies hatched earlier than they would if left undisturbed in ideal conditions (app. C). This shows that daughter colonies have the flexibility to hatch earlier if conditions are not ideal for growth inside their mother colonies. Colonies that were induced to hatch were smaller and had faster swimming speeds than synchronized colonies that hatched naturally the next day (~300 μm/s compared to ~200 μm/s; table B1).

Using the Measurements in the Model to Determine the Physical Limits of the Spherical Design

We now insert back into the model the experimentally measured and calculated parameters. The major result yielded is that the average swimming force per motile cell decreases with colony size, or $f \propto N^{-0.21}$ (sections A and B). If we insert this relation in equation (6a) and (6b), figure 7*A* shows that the size constraint on motility is more stringent. This confirms that the investment in somatic cells has to increase with size for colonies to avoid sinking. Furthermore, the extant species data fit the model quite well. Colonies with larger flagellated cells have a higher flagellar force (f) but not enough to compensate the increase in mass (ΔM; section B). Only the decrease in $\Delta\rho_C$ due to the lower density of large germ cells may ease this constraint (app. C).

We now compute a physical limit on colony size assuming colonies need to be buoyant. If we insert the scaling relations for Nqf, ΔM, and R from the SLR analysis (table 4) in equation (3), we get the swimming speed V_{up} solely as a function of N:

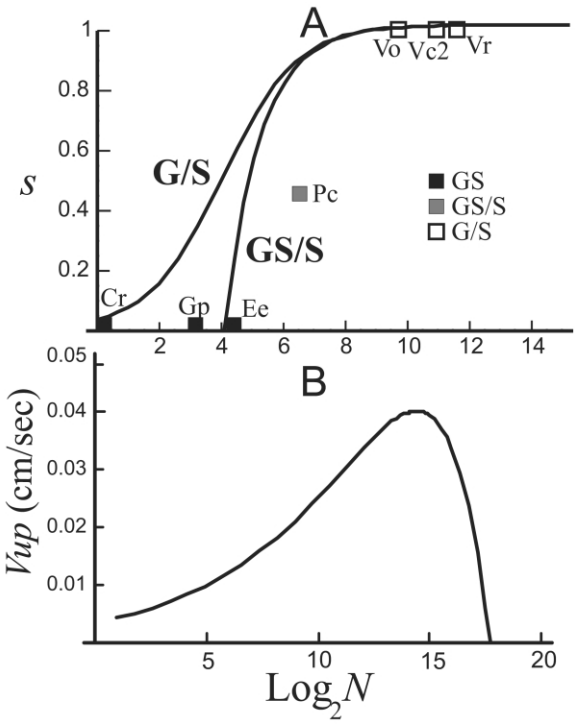

Figure 7: Applying the measurement results to the model. *A*, The major result yielded by the analysis ($f \propto N^{-0.21}$) is inserted in equations (6) to get the proportion *s* of somatic cells needed to avoid sinking as a function of number of cell divisions ($\log_2 N$). The same values in figure 3 are used for $\Delta\rho_C$, r_{in}, r_{Sin}, and r_S. Some of the extant species data is plotted to show how it fits with the model. *Cr*, *Chlamydomonas reinhardtii*; *Gp*, *Gonium pectorale*; *Ee*, *Eudorina elegans*; *Pc*, *Pleodorina californica*; *Vc2*, *Volvox carteri* grown at 1,000 foot-candles (fc); *Vo*, *Volvox obversus*; *Vr*, *Volvox rousseletii*. *GS*, undifferentiated colonies; *GS/S*, soma differentiated colonies; *G/S*, germ-soma differentiated colonies. Although in *D4* colonies reproductive cells start as flagellated cells (e.g., *V. rousseletii*), their flagella have essentially no motility function because they are reabsorbed before the first cell division. Thus, we consider *D4* reproductive cells nonflagellated (G) and *D4* colonies G/S colonies. *B*, The physical limit on colony size assuming colonies need to be buoyant. Scaling relations for *Nqf*, ΔM, and *R* from the SLR analysis (table 4) are inserted in equation (3) to get the swimming speed V_{up} (cm/s) solely as a function of *N* ($\log_2 N$; eq. [6]). $f_{Cr} = 2.4 \times 10^{-7}$ dyn, $\Delta M_{Cr} = 1.01 \times 10^{-11}$ g, and $R_{Cr} = 0.00035$ cm. Note that the hypothetical volvocalean colonies with $N > 2^{18}$ would sink.

$$V_{up} = \frac{1}{6\pi\eta R_{Cr}}(f_{Cr}N^{0.32} - g\Delta M_{Cr}N^{0.61}), \qquad (7)$$

where f_{Cr}, ΔM_{Cr}, and R_{Cr} are the *C. reinhardtii* measured and calculated values used as normalization constants. The flagellar force *f* in *C. reinhardtii* is two to three orders of magnitude higher than the downward gravitational force ($f \sim 10^{-7}$ dyn, $g\Delta M \sim 10^{-9}$ dyn), but as *N* increases, $g\Delta M$ increases proportionally more than *Nqf*, making the colonies sink at a threshold size (fig. 7*B*). If Volvocales need to be buoyant, from figure 7*B* we infer a physical limit on colony size of $N \sim 2^{17}$ for the spheroid design.

Differences between Developmental Programs and Mutant Forms

We now compare differences in the swimming force and speed between developmental programs and mutant forms. When performing MLR on newly hatched colonies (including the mutant forms), we find that the exponent of the relation between V_{up} and *N* becomes 0.26 (SE = 0.02). All mutant colonies, *D2* and *D3* colonies, and nonspherical (*G. pectorale*) colonies have on average a lower V_{up}. *Volvox rousseletii* has on average a higher V_{up} than colonies that do not retain robust cytoplasmatic bridges (table 5; $n = 26$, $r^2 = 0.98$).

The differences in V_{up} among different colony designs reflect the trade-offs between investing in reproduction, increasing colony size (i.e., colony radius), and motility. Since *lag*$^-$ colonies have a larger *R* (fig. 6), their V_{up} is lower due to an increase in drag. In contrast, *V. rousseletii* V_{up} is higher due to a decrease in drag (*R*) and mass (ΔM) because these colonies retain robust cytoplasmatic bridges and their cells are significantly smaller compared to the other species (fig. 6). The *D2* and *D3* colonies have on average a lower V_{up} than the other colonies probably because in proportion they have more reproductive tissue when they hatch, increasing their mass ΔM. We can only speculate that nonspherical *G. pectorale* colonies have a lower V_{up} because a rectangular instead of a spherical design might decrease the flagellar beating efficiency; further studies are needed.

The lower V_{up} of *regA*$^-$ and *gls/regA*$^-$ colonies may be explained by the decrease in the total force produced by these colonies for swimming (fig. 6). Chances are that by being regenerated into reproductive cells, *regA*$^-$ somatic cells invest less in motility and more in growth. When using MLR and correcting for cell size, the exponent of the relation between *Nqf* and *N* becomes 0.78 (SE = 0.02). The *regA*$^-$ and *gls/regA*$^-$ mutant colonies have on average a lower *Nqf*. Evidence thus shows that the *regA*$^-$ mutation negatively affects *f* (table 5; $n = 24$, $r^2 = 0.99$). Table 6 summarizes how motility is affected by developmental programs and mutations that disrupt colony organization.

Discussion

Main Results

A model based on standard hydrodynamics has been developed and experimentally tested in the Volvocales as a means to understand whether the transition from colonies

Table 5: Models selected from the multiple linear regression analysis

$$\log\,[V_{up}] = -2.40 + 0.26\log\,[N] + 0.13Vr - 0.33Gp - 0.09D2 - 0.45D3 - 0.31reg - 0.28gls/reg - 0.18lag$$
$$\log\,[Nqf_{\tilde{a}}] = -3.09 + 0.78\log\,[N] - 0.35reg - 0.43gls/reg$$

Note: Analysis includes mutants and developmental modes. Subscript on *Nqf* means that the response variable was divided by $\tilde{a}$ to correct for cell size. Mixed stepwise regression was used for model selection (probability to enter = 0.25, probability to leave = 0.05). Indicator variables were used for the following nominal factors: light intensity when cultured (600 foot-candles), mutant forms (*regA*$^-$, *gls/regA*$^-$, and *lag*$^-$), same species cultured under two light intensities (*Volvox carteri*), nonspherical colonies (*Gonium pectorale*), developmental programs (*D2*, *D3*, and *D4*), and colonies retaining robust cytoplasmatic bridges (*Volvox rousseletii*).

with unspecialized cells (GS) to multicellular individuals with germ-soma separation (GS/S and G/S) can be explained by the increase in motility requirements of larger colonies.

The main results are as follows. First, because in Volvocales daughter colonies are fully formed inside the mother colonies before they hatch, the enlargement of the reproductive cells (r_{max}) increases the downward gravitational force ($g\Delta M$) of the colony, inhibiting the colony's motility (fig. 3). Overcoming this threshold and avoiding sinking requires the investment in soma and the increase in the N_S/N_R ratio.

Second, the decrease in the collective flagellar beating efficiency with size ($f \propto N^{-0.21}$) further augments the need for investing in somatic cells (fig. 7*A*). It seems that the arrangement of flagellar motors on a sphere of increasing size is not the most efficient design for directional swimming. It should be understood that the force f is an "effective" force per flagellated cell. This force is modified by geometrical factors and reduced by the additional drag caused by the beating of flagella. For example, the flagella beating at the poles probably contribute less to V_{up} than the ones in the equator of the colony. Furthermore, due to the beating of the flagella, the fluid velocity near the colony surface can be higher than the adjacent velocity of a Stokesian sphere moving with V_{up}. This "extra" velocity exerts a downward drag on the colony. By defining f as the "effective" force (i.e., the actual force), we avoid a detailed fluid dynamic analysis beyond the scope of this article. The combined effects of geometry and extra downdrag must be major contributors to the observed power law.

Third, for swimming speeds, the model analysis yields not only the size at which colonies sink but also the size at which motility is optimized. For example, the maximum swimming speed for a GS/S colony with $s = 0.95$ is reached at size $N \sim 128$; the size at which this colony sinks is $N > 512$ (fig. 4*A*). Moreover, in large colonies with a high proportion of somatic cells, germ specialization (i.e., G/S colonies) increases swimming speeds because the nonflagellated germ cells are packaged on the inside and decrease the colony surface area, achieving a hydrodynamically more efficient design (fig. 4*C*). Our experimental data shows that some *Volvox* species can swim at several hundred microns per second (fig. 5), allowing them to migrate in the water column. For example, a colony swimming at 500 μm/s can vertically migrate 2 m/h.

Fourth, as suggested by Koufopanou (1994), in these colonies there seems to be an upper limit for total size. The model shows that there is a size limit for this spherical design given that daughter colonies develop inside the mother colonies and need to stay afloat ($N \sim 2^{17}$; fig. 7*B*). The maximum size that we know of reported in the literature is ~50,000 cells (~2^{16}; e.g., *Volvox barberi* and *Volvox amoeboensis*), in accord with our results (Smith 1944; Kirk 1998).

Finally, the results show trade-offs between investing in reproduction, increasing colony size (i.e., colony radius), and motility (table 6; section D). Increasing colony radius (R) increases drag and decreases swimming speed (e.g., fig. 4*C*). More investment in reproductive tissue to increase fecundity increases the downward gravitational force

Table 6: Net effect that mutant forms and developmental modes have on V_{up}

	Nqf	ΔMg	$6\pi\eta R$	Net effect on V_{up}
D2	0	+	0	−
D3	0	+	0	−
D4	0	0	0	0
Vr	0	−	−	+
reg	−	0	0	−
gls/reg	−	0	0	−
lag	0	0	+	−

Note: An increase (+) in *Nqf* increases V_{up} (+), but an increase in ΔMg and $6\pi\eta R$ decreases V_{up} (−). The *D2* and *D3* colonies have on average a lower V_{up} because in proportion they have more reproductive tissue when they hatch, increasing their mass ΔM. In contrast, when they hatch, *Volvox rousseletii* colonies have a lower R and ΔM and, thus, a higher V_{up} because they retain robust cytoplasmatic bridges and their flagellated and reproductive cells are significantly smaller. The lower V_{up} of *regA*$^-$ and *gls/regA*$^-$ colonies is due to a decrease in the flagellar beating force f of the regenerated somatic cells. The *lag*$^-$ colonies have on average a larger R, which increases the drag and negatively affects V_{up}.

($g\Delta M$) and also decreases swimming speed (e.g., fig. 4*D*). For example, colonies that form larger spheroids (e.g., *lag*$^-$ mutants) or that have higher proportion of reproductive tissue (e.g., *D2*) have lower swimming speeds (fig. 6) or are not able to swim when daughter colonies are formed inside (section B). The trade-off between reproduction and motility is especially clear in *Volvox carteri* mutants: when somatic flagellated cells that are specialized in motility regenerate into reproductive cells (*regA*$^-$), they have a lower flagellar beating force, indicating that shifting limiting resources within the cell to reproduction decreases swimming capabilities (fig. 6; section D, wild-type $f \sim 8 \times 10^{-8}$ dyn, *regA*$^-$ $f \sim 5 \times 10^{-8}$ dyn, *gls*/*regA*$^-$ $f \sim 3 \times 10^{-8}$ dyn).

Relation of Results to the Evolution of Multicellularity and Natural History

Based on our results, we believe that the transition from undifferentiated to germ-soma differentiated Volvocales can be best explained as a consequence of the constraints and opportunities given by motility as colonies increase in size. We provide strong evidence that producing increasingly larger volvocalean colonies places a significant cost on motility and, therefore, on colony viability that is compensated for by increasing cell specialization. For colony size to increase, a specialized and sterile soma has to evolve, and the N_S/N_R ratio has to increase as colony size increases to keep colonies buoyant and motile while reproductive cells divide and develop. Even within the same species, larger colonies invest in a higher proportion of somatic cells to maintain similar swimming speeds. For example, when *V. carteri* is grown at different light intensities (600 and 1,000 fc), colony size is larger at the higher light intensity and so is the ratio of somatic to reproductive cells (difference in the N_S/N_R ratio = 45, SE = 12, *t*-test; $P = .001$, $n = 40$; table 3), but swimming speeds of newly hatched colonies for the two populations do not differ (*t*-test; $P = .98$, $n = 70$; table B1). Furthermore, a specialized nonflagellated germ cell can presumably invest more resources in reproduction. Therefore, increased specialization (i.e., GS/S to G/S colonies) enhances both the motility (by decreasing colony drag as we have shown) and productivity of the larger colonies.

Unicellular and multicellular forms of Volvocales coexist in transient, quiet bodies of water or in large and deep permanent eutrophic lakes (during early summer blooms). Most of what is known about their ecology is from the latter situation (Kirk 1998). How much of the colonies' collective flagellar beating is invested in translocation and/or nutrient uptake probably depends on the environment where they live. For example, in large ponds, motility might be crucial to perform daily migration in the water column to best use resources (such as light, nitrogen, phosphorus) that are heterogeneously distributed, both spatially (surface vs. bottom) and temporally (day vs. night; Sommer and Gliwicz 1986). In contrast, in quiescent, small, shallow puddles, motility might not be as important, but flagellar beating might be crucial for enhancing nutrient uptake by local mixing of fluid (Niklas 1994, 2000; Solari et al. 2006).

Our results suggest that the different colony designs of *Volvox* species are adaptations to different environments with different selective pressures on motility and reproduction (i.e., permanent deep lakes vs. shallow transient ponds, high vs. low predation pressure). Some species invest more in reproduction over motility (*D2* vs. *D1* colonies; fig. 4*D*), increasing the proportion of germ tissue and overall colony mass. We speculate that these colonies should have an advantage when motility needs are reduced such as in a shallow transient pond. Also, some colonies prioritize size (i.e., colony radius) over motility (*Volvox gigas* vs. *Volvox rousseletii*), increasing their radius by increasing the amount of ECM. We speculate that these colonies should have an advantage when the predation pressure is significant or storage of nutrients in the ECM is important. As colonies increase in radius, they can be ingested by a smaller variety of zooplankton grazers and predators (Porter 1977; Morgan 1980; Pentecost 1983; Reynolds 1984). In contrast, *Volvox* colonies that have a smaller initial hatching size and a lower proportion of reproductive tissue have higher motility capabilities (e.g., *V. rousseletii* and prematurely hatched *V. carteri*; figs. 5, 6; table B1). We expect these colonies to have an advantage in environments where the need for motility is high, such as in stratified deep permanent lakes. In conclusion, we infer that the trade-offs between fecundity, predation pressure, and motility in different environments are important selective factors affecting hatching size and colony design. The next necessary step is to perform detailed ecological studies.

Relation of the Hydrodynamic Model to Previous Work

Undifferentiated (GS) colonies face motility problems at a small size ($N = 32$) as a result of the "flagellation constraint" (explained above; Koufopanou 1994). Nonetheless, even in the absence of the flagellation constraint, both the model (figs. 3, 4) and our data (e.g., *gls*/*regA*$^-$ mutant; table B1) show that larger GS colonies would sink anyway unless they invest in somatic tissue and the associated ECM (i.e., GS/S and G/S colonies). Moreover, the hydrodynamic model gives strong evidence that the investment in soma has to increase with colony size for the colonies to stay afloat and motile, in agreement with Koufopanou's (1994) conclusions.

The "source-sink" hypothesis argues that larger cell-differentiated colonies with a higher N_S/N_R ratio are more efficient in nutrient uptake and storage, especially in eutrophic conditions (Bell 1985; Koufopanou and Bell 1993; Kirk 1998, 2003). The benefits of the source-sink hypothesis developed by Bell (1985) to explain germ-soma separation in Volvocales could only be enjoyed if ways to maintain the motility of these large colonies (i.e., through the evolution of a sterile but permanently motile soma) have already evolved. This hypothesis was not intended to take into account the viability constraints imposed by these organisms' peculiar type of development, which disrupts the motility of the colonies as size increases. Furthermore, in order for the source-sink hypothesis to work, we believe that the boundary layer stirring caused by the collective flagellar beating of somatic cells is important to keep a high nutrient gradient outside the ECM (Niklas 1994; 2000). In work presented elsewhere, we report the importance of flagellar mixing and transport to enhance nutrient uptake and increase the productivity of large *Volvox* colonies (Solari 2005; Solari et al. 2006). Nevertheless, the geometrically required evolution of the ECM for the addition of flagellated cells in the surface of the colony concurrently benefits nutrient uptake and storage as stated by the source-sink hypothesis.

The quantitative measurements and associated hydrodynamic modeling provided here complement and expand the existing hypotheses and with them provide a fuller account of the remarkable transitions in individuality observed in this lineage. We expect that more detailed extensions of the quantitative hydrodynamic analysis will provide generally applicable insights into the origins and utility of enlargement, multicellularity, and specialization. To summarize, we argue that the costs of reproducing a larger organism can be an important driving force for the evolution of life-history traits and increased cell specialization. Each degree of specialization and differentiation may counteract the higher costs associated with larger size by increasing the viability and/or the productivity of the larger organism, therefore allowing it to reach fitness levels impossible to attain without increased complexity.

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APPENDIX A

Model Equations

The difference in mass between the colony and the water displaced, $\Delta M = 4/3\pi R^3\Delta\rho$, can be stated as the sum of the difference in mass between the cells and the water they displace and the difference in mass between the extracellular matrix (ECM) and the water it displaces. Assuming that colonies and cells are spheres,

$$\Delta M = \frac{4}{3}\pi(R^3\Delta\rho_{\mathrm{ECM}} + N\{[(1-s)r^3\Delta\rho_{\mathrm{G}} + sr_{\mathrm{S}}^3\Delta\rho_{\mathrm{S}}] - [(1-s)r^3 + sr_{\mathrm{S}}^3]\Delta\rho_{\mathrm{ECM}}\}), \tag{A1}$$

where r and r_S are the radii of the reproductive, GS or G, and S cells, respectively. The $\Delta\rho_G$, $\Delta\rho_S$, and $\Delta\rho_{ECM}$ are the difference in density between reproductive cells, S cells, the ECM, and water. When $s = 0$, colonies only have undifferentiated GS cells, as in *Eudorina*. As a first approximation we assume that $\rho_G = \rho_S$, and, because of the apparently aqueous nature of the ECM, $\rho_{ECM} = \rho_W$. Thus, ΔM becomes the product of N, $\bar{u}$ (the weighted average of the cell volume), and $\Delta\rho_C$ (the average difference in density between cells and water), yielding

$$\Delta M \approx \frac{4}{3}\pi[(1-s)r^3 + sr_{\mathrm{S}}^3]\Delta\rho_{\mathrm{C}}N \approx \frac{4}{3}\pi\bar{u}\Delta\rho_{\mathrm{C}}N. \tag{A2}$$

Colony radius R depends on the number of flagellated cells Nq, composed of GS and/or S cells, and on the area between cells. We model flagellated cells as circles arrayed on the sphere surface, A being a cell concentration term correcting for the intercellular surface area. Then,

$$Nq = \frac{4\pi R^2}{\pi\{[1-(s/q)]r^2 + (s/q)r_{\mathrm{S}}^2\} + \pi A}. \tag{A3}$$

For GS colonies, $s/q = 0$ because $s = 0$ (e.g., *Eudorina*); for GS/S colonies, $s/q = s$ because $q = 1$ (e.g., *Pleodorina*); and for G/S colonies, $s/q = 1$ because $s = q$ (e.g., *Volvox carteri*). We then have

$$R \approx \frac{1}{2}\left[\left(1 - \frac{s}{q}\right)r^2 + \frac{s}{q}r_S^2 + A\right]^{1/2} q^{1/2}N^{1/2}$$

$$\approx \frac{1}{2}(\bar{a} + A)^{1/2}q^{1/2}N^{1/2}, \quad \text{(A4)}$$

where $\bar{a}$ is the weighted average of the flagellated cell area.

Finally, we assume that the size r_{max} that a colony's reproductive cell with palintomic development has to reach to produce a colony of the same type is a function of the number, initial size, and type of cells in that colony:

$$N \approx \frac{(4/3)\pi r_{max}^3}{(4/3)\pi[(1 - s)r_{in}^3 + sr_{Sin}^3]}. \quad \text{(A5)}$$

Solving for r_{max},

$$r_{max} \approx [(1 - s)r_{in}^3 + sr_{Sin}^3]^{1/3}N^{1/3}, \quad \text{(A6)}$$

where r_{in} and r_{Sin} are the initial radii of the reproductive, GS or G, and S cells, respectively. The data confirms that $r_{max} \propto N^{1/3}$, as used in the model. When analyzing the maximum radius measured on reproductive cells before the division phase versus N, even without correcting for initial cell size (not measured), SLR yields $r_{max} \propto N^{0.31}$, not significantly different from 0.33 (SE = 0.07, $n = 8$, $r^2 = 0.87$; table B1).

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