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Fitness trade-offs and developmental constraints in the evolution of soma: an experimental study in a volvocine alga

Matthew D. Herron, Susma Ghimire, Conner R. Vinikoor, Richard E. Michod

Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, USA

Abstract

Background: The evolution of mortal somatic cells was a critical step in the evolution of complex body plans and the major radiations of multicellular life. In the volvocine green algae, somatic cells are hypothesized to mitigate an increasing cost of reproduction as colony size increases, primarily by providing motility to the colony during reproduction.

Questions: Does selection on colony size cause an evolutionary response in proportion of somatic cells? Does the effect of selection on colony size differ in environments that differ in the importance of motility?

Methods: We subjected an outcrossed population of the volvocine alga *Pleodorina starrii* to selection on colony size in still and mixed environments. After approximately 40 generations with periodic selection, we estimated the relationship between colony size and proportion of soma in evolved colonies from both environments.

Results: In the largest size category, colonies selected in the still environment (in which motility is hypothesized to be more important) had a higher proportion of soma than those from the mixed environment. Within-strain variation in cell number was surprisingly large: up to 16-fold for some genotypes. The positive among-species relationship between colony size and proportion of soma was paralleled within the larger (16- to 64-celled) colonies of *P. starrii*, but not within the smaller (4- and 8-celled) colonies, which had the highest proportions of soma, suggesting the existence of an evolutionary constraint preventing optimization of soma in the smallest size classes.

Keywords

cellular differentiation; experimental evolution; multicellularity; *Pleodorina*; Volvocaceae

INTRODUCTION

The evolution of multicellular organisms has had a major impact on the ecology and evolution of life on Earth. A universal feature of the familiar, macroscopic multicellular groups (plants, animals, fungi, red algae, brown algae, etc.) is cellular differentiation, the specialization of cells into functional types. One of the most basic distinctions between cell types is between the potentially immortal cells of the germ line and the mortal cells of the

somatic tissues. The evolution of terminally differentiated somatic cells was a critical step in the evolution of complex body plans and the major radiations of multicellular life (Buss, 1987; Michod, 2006), and its fundamental importance to the process of evolution was recognized as far back as Weismann (Weismann, 1889). Somatic cells are an evolutionary dead end; only mutations affecting the germ cells have any chance of being passed on to future generations. The evolution of soma is a particularly challenging problem in evolutionary biology, as somatic cells (by definition) give up reproduction and, therefore, their kin-selected benefits must outweigh their direct fitness costs.

The volvocine green algae are a model system for the origins of multicellularity and cellular differentiation. These motile, facultatively sexual, haploid eukaryotes display a diverse range of body sizes and degrees of specialization, including single-celled forms and colonial forms with and without germ-soma differentiation (Iyengar and Desikachary, 1981; Ettl, 1983; Nozaki, 2003). Colonial volvocine algae are generally small clumps or sheets of up to 32 cells, such as *Gonium*, or spheres with cells arranged on the periphery, such as *Eudorina*, *Pleodorina*, and *Volvox* (Iyengar and Desikachary, 1981; Ettl, 1983). Soma has arisen at least three times within this group (Herron and Michod, 2008) and can represent a small (<15%, *Astrephomene*), intermediate (~10–50%, *Pleodorina*) or large (>99%, *Volvox*) proportion of the cells in a colony (Iyengar and Desikachary, 1981; Ettl, 1983; Nozaki, 2003). Soma in this context does not imply the sort of early segregation of somatic and germ lines seen in animals, but only that a subset of cells are specialized for functions other than reproduction.

Among volvocine species, two trends are apparent in the relationship between size and level of specialization: only species with ~32 or more cells have cellular specialization, and, within the germ-soma specialized forms, larger species have higher proportions of somatic cells (Koufopanou, 1994). The evolution of soma in volvocine algae has been hypothesized to result from within-species trade-offs between motility and fecundity (Koufopanou, 1994; Solari et al., 2006), as large colonies require somatic cells to maintain motility (see below). The trend of larger species having higher proportions of soma is consistent with this hypothesis; however, trends among species can differ substantially from those within species, and it is trends within species that affect adaptive change. The central question of this study is: Are the trends within a species consistent with the trade-offs between motility and fecundity that are hypothesized to have driven this evolutionary transition? Towards this end, we studied colonies of the volvocine alga *Pleodorina starrii* to address the following questions: How is proportion of soma related to colony size? Does colony size or proportion of soma differ between environments in which motility is more or less important?

In all colonial volvocine algae, asexual reproduction occurs through autocolony formation, in which each reproductive cell (every cell in species without germ-soma differentiation) forms a new ‘daughter’ colony. In most species (including *Pleodorina starrii*, the subject of this study), daughter colonies develop through palintomy, a process in which cells grow to several times their original size and then divide multiple times with little or no growth between successive divisions (Sleigh, 1989; Desnitski, 1992). All cell divisions occur early in development, so the final cell number of daughter colonies is fixed before they are released from the mother colony. In *P. starrii*, cells divide symmetrically, so all cells are the

same size early in development (Nozaki et al., 2006). As colonies mature, reproductive cells grow faster than somatic cells, so mature colonies can be recognized by the presence of large (reproductive) and small (somatic) cells. In species with germ-soma differentiation, somatic cells die shortly after the release of daughter colonies. All subsequent growth of daughter colonies is through increases in cell size and in the volume of extracellular matrix.

In the volvocine algae, sterile somatic cells are thought to have evolved in response to trade-offs between motility and fecundity, i.e. a cost of reproduction in terms of motility (Koufopanou, 1994; Michod, 2006; Solari et al., 2006). Motility is an important component of viability in volvocine algae because it prevents sinking and allows phototaxis, gravitaxis (Solari et al., 2006), and (in some species) chemotaxis (Hoops et al., 2002). In addition to providing motility, flagellar activity is important for the efficient exchange of nutrients, metabolites, and waste (Michod et al., 2006; Short et al., 2006; Solari et al., 2006). The spatial organization of a spheroidal cell group dictates a decrease in surface area to volume ratio as size increases. This can adversely affect the type and extent of interactions with the environment, diminishing, or perhaps exhausting, aggregate resource availability, with profound consequences for metabolism, growth rate, viability, and fecundity. The mixing that results from flagellar activity helps to overcome this effect by preventing the boundary layer of medium surrounding the colony from becoming depleted of nutrients (Short et al., 2006; Solari et al., 2006, 2007).

The cost of reproduction is acute in larger colonies because of two developmental constraints: the ‘flagellation constraint’ and the particular mode of cell division – palintomy or multiple fission. The flagellation constraint sets an approximate upper limit of about five for the number of times a cell can divide while maintaining active flagella (Koufopanou, 1994). Because of a coherent cell wall, the position of the flagella is fixed, and the basal bodies cannot move laterally to take the position expected for centrioles during cell division while remaining attached to the flagella (Koufopanou, 1994). Because the basal bodies must detach from the flagella during cell division, cells can only maintain flagellar activity through 4–5 rounds of cell division [flagella detached from their basal bodies continue to beat for this length of time (Koufopanou, 1994)]. In most species, including *P. starrii* (Nozaki et al., 2006), each cell, whether reproductive or somatic, bears two flagella. Soma may allow colonies of >32 ($= 2^5$) cells to remain motile while reproductive cells undergo mitosis; this is consistent with the size distribution of colonies with soma (all species with >32 cells are germ-soma specialized; all species with <32 are undifferentiated). Because the flagellation constraint affects colonies of >32 cells, the trade-off hypothesis predicts that the motility benefits of soma will be most evident in the largest (64-celled) colonies.

Multiple fission requires the growth of large germ cells to form large daughter colonies, since no growth occurs between cell divisions. Under this type of development, a cell needs to grow 2^d -fold in order to divide d times and produce a colony with 2^d cells. This growth increases the mass and size of a colony during the reproductive phase, which in turn negatively impacts motility and exchange of nutrients and waste with the environment (Solari et al., 2006). Drag increases in proportion to colony diameter (Solari et al., 2006), and the palintomic program of cell division requires large species to have large germ cells.

Cytoplasm is denser than water (Solari et al., 2006), so large germ cells greatly increase the negative buoyancy of colonies.

As colonies evolve larger sizes, then, they face several challenges related to motility. Reproductive cells in colonies larger than ~32 cells cannot maintain flagellar beating throughout cell division. Somatic cells help to overcome this restriction by continuing flagellar activity while reproductive cells divide. Negative buoyancy increases because of the larger germ cells needed to produce larger colonies, and drag increases with the diameter of the colony. Somatic cells mitigate both of these costs by virtue of their small size, which contributes less to negative buoyancy and allows a large number of cells (and flagella) to occupy a small area on the surface of the colony. For all of these reasons, it has been hypothesized that the cost of reproduction to viability increases with colony size in the volvocine algae and that this increasing cost selects for increasing soma as the colony size increases beyond 32 cells (Koufopanou, 1994; Solari et al., 2006, 2007). In *P. starrii*, cell numbers range from 4 to 64, so only the 64-celled colonies are expected to need somatic cells to maintain motility. Hence it is the 64-celled colonies that present the most critical test of the fitness trade-off hypothesis for the evolution of soma.

To estimate the relationship between colony size and proportion of soma, we measured genetic correlations among colony diameter, cell number, and proportion of soma in a genetically heterogeneous population of *P. starrii*. In order to increase the range of sizes, we subjected outcrossed populations to selection for increasing and decreasing colony size. The primary goal of outcrossing and selection was to increase genetic variation in colony size in order to make genetic correlations with other traits easier to measure. We chose *P. starrii* for this experiment because it has an intermediate proportion of soma, allowing for the possibility that this trait could change in either direction, and because it is heterothallic, ensuring that any sexually produced offspring are outcrossed and that the progeny of isolated colonies are clonal. Both treatments, along with an unselected control, were replicated in environments in which motility was hypothesized to be more important (still environment) or less important (mixed environment). We measured colony diameter, cell number, and proportion of soma in clonal lines isolated after the selective treatments to estimate genetic values of these traits.

MATERIALS AND METHODS

Outcrossing and artificial selection

To create an initial pool of genetic variability, experimental cultures were established from sexually produced offspring of eight *P. starrii* strains obtained from the National Institute of Environmental Studies in Japan. Male and female strains were crossed to produce spores and germinated using a modified version of the protocol described in Goldstein (1964) (see evolutionary-ecology.com/data/2917Appendix.pdf for details). Since *P. starrii* is haploid, the initial outcrossed population could have included up to five alleles at a given locus (at most one for each independently collected parental strain; see [2917Appendix.pdf](http://evolutionary-ecology.com/data/2917Appendix.pdf)). The purpose of crossing strains to found the experimental populations was to recombine the alleles present in a large number of different combinations. The starting population included nearly 4000 isolates from all six F1 crosses, six of the seven possible F1 × P back-crosses, and two F2

crosses (2917Appendix.pdf). Colonies resulting from these crosses were isolated in 96-well tissue culture plates in 300 μ L standard *Volvox* medium (Kirk and Kirk, 1983).

The artificial selection experiment included six treatments (Fig. 1): selection for large size (hereafter ‘plus’), selection for small size (‘minus’), and an unselected control group, each with and without stirring of the medium (‘mixed’ and ‘unmixed’). Each treatment initially included 21 replicate cultures seeded at the start of the experiment with $\sim 10^4$ colonies from a common stock of sexually produced *P. starii* (2917Appendix.pdf).

Experimental populations were grown in batch culture at 25°C on a 16 hour light/8 hour dark cycle, and selection was imposed 11 times at 7-day intervals (~ 3 asexual generations). Under these conditions, only asexual reproduction occurs (Nozaki et al., 2006). Because a single volvocine colony can produce many offspring, generations in this experiment are not equivalent to colony doubling times. For example, a population consisting of 32-celled *P. starii* colonies, each with 20 reproductive and 12 somatic cells, would increase ~ 20 -fold each generation, or ~ 8000 -fold in 7 days, under ideal growth conditions.

Test tubes were placed in random order (still and mixed treatments randomized separately) in a single row 10 cm from 24 fluorescent lights (17W, 4100K). Selection was imposed by passing the culture medium through phosphor bronze filters (Mini-Sieve Micro Sieve Set, Bel-Art Products, Pequannock, NJ). Plus and control treatments used #170 mesh filters (88 μ m openings); minus treatments used #230 mesh filters (63 μ m openings). Colonies that passed through the filter (for the small size treatment) or were retained (for the large size treatment) were used to inoculate fresh medium (Fig. 3). Control tubes were inoculated with a mixture of colonies that passed through the filter and those that were retained. Mixing was achieved by gentle agitation at 160 rpm on orbital shakers.

Trait measurements

To estimate genetic values of traits, we established six isogenic cultures from each experimental population. After 11 rounds of asexual reproduction and selection, we isolated single colonies from each population and grew them in 330 μ L SVM in 96-well plates. Clonal progeny descending from one of these isolated colonies is hereafter referred to as an isolate line. Isolate lines were maintained at 20°C, 16 hours/8 hours light/dark, and 30 μ L transferred to fresh medium every 7 days. Six weeks after establishment of isolate lines, a thermostat failure caused the incubation temperature to increase to 48°C for an unknown length of time (less than 8 hours). Many of the experimental isolate lines did not recover, so morphological measurements were made on five isolate lines from each of eight replicates per combination of treatment \times environment (3 treatments \times 2 environments \times 8 replicates \times 5 isolates = 240 isolate lines).

Since each isolate line was genetically homogeneous, quantitative genetics attributes within-isolate line variation to environmental, maternal, and/or error components. All trait measurements were made on colonies propagated in 4.8 mL fresh, still medium in 6-well tissue culture plates for 7 days (~ 3 asexual generations) to minimize environmental and maternal effects (Fig. 1). For asexual reproducers, within-genotype means are (by definition) genetic values. Heterothally ensures that isolate lines reproduce clonally (since a given

isolate line is made up of colonies of one sex), so the average value of a trait within an isolate line was considered the genetic value.

Altogether, 800 μL of colonies growing at low density were pipetted into a 9-well glass spot plate (Corning, Inc., Corning, NY) and stained with 2 μL iodine. After settling for ~ 1 hour, all or nearly all colonies could be aspirated into 18 μL , which was transferred to a glass microscope slide. Slides were photographed on an 8×12 or 12×12 grid pattern at $150\times$ magnification at a resolution of 3840×3072 pixels on a Nikon DS-Ri1 digital camera mounted on a Nikon Eclipse Ti inverted microscope. Morphological measurements were made using Nikon NIS-Elements AR Imaging Software version 3.00 (Build 550).

Analyses

Colony diameter was estimated as the average of the longest axis and the longest perpendicular to the longest axis. Cell size was measured as an area, and cell diameter was the equivalent diameter for the measured area ($\sqrt{4A/\pi}$). Only mature colonies, those in which cell types could be distinguished as described below, but which had not begun to reproduce, were measured for colony diameter. Cell numbers were linearized as the logarithm with base 2, i.e. the number of rounds of cell division required to produce the observed number of cells. When not specified, comparisons among selective treatments or between still and mixed environments are one-way analyses of variance (ANOVAs), and P -values are reported for Tukey *post-hoc* comparisons (all pairwise comparisons tested).

Somatic and reproductive cells were identified by a combination of methods. For mature (but non-reproductive) colonies, equivalent diameters of cells were sorted into two categories using K -means clustering with the Hartigan-Wong algorithm (Hartigan, 1975; Hartigan and Wong, 1979) in R version 2.9.2 (R Development Core Team, 2009). K -means clustering was deemed to have accurately classified cell types if the ratio of diameters of the smallest germ cell to the largest somatic cell was the largest ratio of any two consecutively ranked cells (i.e. the largest 'gap' in the ordered list of diameters was between, not within, the categories identified by K -means clustering). Colonies that did not meet this requirement were considered immature and not counted. For reproductive colonies (those whose reproductive cells had begun dividing), somatic cells and embryos (counted as reproductive cells) were counted manually.

The distribution of cell numbers in each isolate line was estimated by tabulating 200 colonies (in a few cases, fewer than 200 colonies were photographed; in these cases, all photographed colonies were counted). For comparisons across isolate lines, weighted averages of colony diameter, cell number, and proportion of soma were estimated by summing the product of the mean value for colonies of a given cell number within an isolate line and the proportion of colonies with that cell number (i.e. $\Sigma[\text{mean trait value of } n\text{-celled colonies} \times \text{proportion of colonies with } n \text{ cells}]$).

RESULTS

Variation in colony size

Summary statistics for colony size are presented in Tables 1 and 2. We measured two components of colony ‘size’: number of cells and colony diameter. The two components have the potential to vary independently, and therefore to respond differently to selection, because of differences in cell size and in the amount of volume-filling extracellular matrix. We thus consider each component separately, beginning with cell number. For analyses involving cell number, it is helpful to bear in mind that volvocine cell numbers are restricted to powers of 2. References to colonies ‘from’ the mixed or the still environment indicate the environment in which selection on body size was imposed; all measured colonies were grown in the same environment (without mixing; see Methods).

Weighted average of colony diameter was significantly higher in colonies from the still environment than the mixed one ($P = 0.0007$; two-way ANOVA: colony diameter by selective treatment + environment, no significant interaction between treatment and environment) but did not differ significantly among selective treatments (plus, control, and minus) in either environment (Table 1). Although selection by filtration was not consistently effective in changing colony diameters, it was effective in increasing variation: genetic values of colony diameter for colonies with the same number of cells varied across isolate lines by between 2.4-fold and 6.2-fold (e.g. 71 μm to 172 μm in 32-celled colonies), compared with 1.4- and 1.5-fold in parental strains (e.g. 98 μm to 148 μm in 32-celled colonies).

Because the trade-off hypothesis predicts different effects on colonies with different numbers of cells, we analysed the evolved populations within cell-number categories. Pooled across environments, genetic values of colony diameter differed significantly among treatments only in 32-celled colonies (control > minus; $P = 0.029$). The average genetic value of colony diameter was larger in colonies from the still environment than those from the mixed one for every cell number, significantly so for 8- and 64-celled colonies (Table 2; one-way ANOVA: $P = 0.042$ for 8-celled colonies; $P = 0.010$ for 64-celled colonies).

Most experimental isolate lines, and all five parental strains, produced colonies of two or more cell numbers (e.g. 32- and 64-celled colonies) when grown at low density in fresh, still medium. In fact, a substantial number of isolate lines (42 of 240) contained 4-, 8-, 16-, 32-, and 64-celled colonies. Genetic values (averages within isolate lines measured in the same environment) of cell number varied over a three-fold range (21.2 to 64.0), compared with ~1.6-fold in the parental strains (24.0 to 37.7). Modal cell number for most isolate lines (210/240), and for all five parental strains, was 32; the remaining isolate lines had a modal cell number of 64. Of the 30 isolate lines with a modal cell number of 64, 25 were from the still environment.

We compared genetic values of cell number across isolate lines as weighted averages, as described in the Methods. Cell number is an ordinal measure and would normally be better treated using non-parametric statistics; however, weighted average of cell number is continuous, and so we compared these measures across isolate lines using standard

ANOVAs. Weighted average cell number was significantly higher in the minus treatments than in the control or plus treatments ($P = 0.006$ for both comparisons after Bonferroni adjustment) and higher in the still environment than in the mixed one ($P = 0.0031$; two-way ANOVA: $\log_2[\text{cell number}]$ by selective treatment + environment, no significant interaction between treatment and environment). One-way ANOVAs showed that there were significant differences in weighted average of cell number among selective treatments in both environments: in the still environment, minus > plus ($P = 0.049$) and minus > control ($P = 0.013$); in the mixed environment, minus > control ($P = 0.001$).

Variation in proportion soma

Summary statistics for proportion of soma are given in Tables 3 and 4. The average number of somatic cells in colonies of a given cell number varied across isolate lines by over 4-fold (e.g. 3.0–13.3 in 32-celled colonies), much more than that in the parental strains (e.g. 10.2–11.9 in 32-celled colonies). Modal numbers of somatic cells in colonies from both environments were 2 for 4-celled colonies, 4 for 8- and 16-celled colonies, and 12 for 32-celled colonies (Fig. 2A–D). The 64-celled colonies from the mixed environment had a mode of 24 somatic cells; those from the still environment had a mode of 28 somatic cells (Fig. 2E). Numbers of somatic cells were significantly non-normally distributed within each cell number category (Kolmogorov-Smirnov test, Shapiro-Wilk normality test; all $P < 0.0001$). Numbers of somatic cells in 16-celled colonies were markedly left-skewed (skew = 2.44; $P < 0.0001$), while those in 32-celled colonies were strongly right-skewed (skew = -1.65; $P < 0.0001$).

The weighted average of proportion of soma was affected by an interaction between treatment and environment (two-way ANOVA on arcsine square root transformed data; $P = 0.0001$). In the mixed environment, the weighted average of the minus treatment (0.35 ± 0.04 ; mean \pm standard deviation) was lower than that of the plus treatment (0.37 ± 0.01 ; one-way ANOVA on arcsine square root transformed data; $P = 0.012$) but statistically indistinguishable from that of the control (0.36 ± 0.02 ; $P = 0.092$). In the still environment, both plus and minus treatments had higher weighted average proportions of soma (0.36 ± 0.04 , 0.37 ± 0.04 , respectively) than the control (0.33 ± 0.07 ; $P = 0.007$).

Among colonies of the same cell number, proportions of soma differed between colonies from the mixed and still environments but not among selective treatments (Table 4). Significant differences in proportion of soma were found in 8-celled colonies (still > mixed; one-way ANOVA: $P = 0.024$), 32-celled colonies (mixed > still; $P = 0.034$), and 64-celled colonies (still > mixed; $P = 0.0005$).

Arcsine square root transformed genetic values of proportion of soma were positively correlated across cell number categories (i.e. isolate lines with high proportions of soma in one cell number category tended to have high proportions in the other cell number categories). Correlation coefficients were positive (some only slightly so) for all ten pairwise comparisons (4-celled colonies vs. 8-celled colonies, etc.; Table 5). Before correction for multiple comparisons, the correlations were significant (Spearman's rank correlation, $P < 0.01$) between 8- and 64-celled colonies ($P = 0.003$), 16- and 32-celled colonies ($P = 0.009$),

16- and 64-celled colonies ($P < 0.0001$), and between 32- and 64-celled colonies ($P < 0.0001$).

Relationship of soma with colony size

In colonies from both environments, mean proportion of soma was highest (~0.5) in 4-celled and 8-celled colonies and lowest (~0.29) in 16-celled colonies (Table 4). In the mixed environment, both 32-celled and 64-celled colonies had a similar mean proportion of soma (~0.36; $P = 1.00$ after Bonferroni correction; Table 4); in the still environment, mean proportion of soma was significantly higher in 64-celled colonies (0.39) than in 32-celled colonies (0.34; $P = 0.0005$ after Bonferroni correction; Table 4).

In terms of weighted averages, proportion soma was positively but weakly correlated with colony diameter in both the mixed ($R^2 = 0.046$, $P = 0.019$) and still ($R^2 = 0.089$, $P = 0.001$) environments (Fig. 3A, B). The relationship between the weighted average of $\log_2(\text{cell number})$ and of proportion soma was curvilinear, with intermediate cell numbers averaging the lowest proportions of soma (Fig. 3C, D). Stepwise Akaike Information Criterion [AIC (Akaike, 1974)] and F -tests ($P < 0.005$) indicated that binomial regressions provided better fits than linear regressions within each environment and across environments.

Based on this evidence for relationships of soma with colony diameter and cell number, we performed a multiple regression analysis on genetic values of proportion soma. The model for this analysis was

$$y = \bar{y} + ax_1 + bx_1^2 + cx_2,$$

where y = genetic value of proportion soma, x_1 = genetic value of $\log_2(\text{cell number})$, and x_2 = genetic value of colony diameter. All three terms were significant (F -test; $P < 0.011$) and collectively explained 0.44 of the variance in proportion soma (multiple R^2). The final model was

$$y = 1.309 - 0.420x_1 + 0.043x_1^2 + 0.0003x_2.$$

Stepwise AIC indicated inclusion of all three interaction terms, which increased multiple R^2 to 0.56.

DISCUSSION

Investing limited resources into soma means investing less into reproduction. In palintomic volvocine algae such as *P. starrii*, this cost is particularly easy to measure: the total number of cells is fixed early in development, and each cell that differentiates as soma reduces the number of reproductive cells, and the number of potential offspring, by one. For the cell, giving up all of one's reproduction to benefit the group is the ultimate form of altruism; understanding the evolution of soma therefore has implications not only for the evolution of multicellularity and cellular specialization, but for the evolution of altruism and cooperation in general.

Response to selection

The main goal of selection by filtration was to generate a larger range of variation than was present in our parental strains so as to measure genetic correlations in trait values. The combination of outcrossing and selection was effective in accomplishing this, resulting in substantially increased ranges (relative to the parental strains) of genetic values of cell number, colony diameter, and proportion soma. With regard to changing mean colony size, filtration appears to have been inconsistently effective. The average diameter of all colonies in an isolate line (weighted average) did not differ significantly among selective treatments in either environment. The lack of response to selection by filtration was likely due to the unforeseen magnitude of within-strain variation in size: if all of the genotypes within an experimental replicate produce a range of colony sizes, some colonies from every genotype are likely to be above (or below) the cut-off imposed by filtration. This non-genetic variation was compounded by the lack of synchronization in the culture conditions, which led to a mixture of mature and immature colonies being present at the time of selection on size.

The environment in which selection took place had a more consistent effect on colony size than selection by filtration, as colony size was larger by several measures in colonies from the still environment. These differences may reflect adaptation to either or both environments. Since all measurements were made on colonies grown in still medium, isolate lines subjected to selection in the still environment may have been better able to grow under measurement conditions. If so, this suggests a role for flagellar activity in colony growth. The relatively homogeneous conditions of the mixed environment were designed to eliminate the need for motility; in the still environment, colonies needed a positive upward swimming speed to stay near the surface where gas exchange occurred. The movement of medium in the mixed environment may also have reduced the need for flagellar stirring of the boundary layer. Colonies adapted to these conditions may have been smaller in the still conditions in which they were measured because they were unable to remain near the surface, because of insufficient nutrient uptake, or both. Such an effect would be most pronounced in the largest colonies, and in fact 64-celled colonies from the still environment had both a larger diameter and a higher proportion of soma than those from the mixed environment.

Relationship of soma to colony size

The most obvious pattern in proportion of soma is among colonies with different numbers of cells, with a mode of 0.5 for 4- and 8-celled colonies, 0.25 for 16-celled colonies, 0.375 for 32-celled colonies, and 0.375 (mixed environment) or 0.438 (still environment) for 64-celled colonies. The curvilinear relationship between the weighted averages of soma and cell number reflects the parallel relationship in genetic values, i.e. proportions of soma are highest in colonies with the largest and smallest cell numbers.

The most striking difference in soma between the still and mixed environment was in the 64-celled colonies, as predicted by the fitness trade-off hypothesis. Both the mean genetic value (24.7 somatic cells vs. 23.3) and the modal value (28 vs. 24) were higher in 64-celled colonies from the still treatment than in those from the mixed one. This difference in soma supports the hypothesis that soma evolved to mitigate trade-offs between motility and

fecundity that become especially acute in colonies of >32 cells, because of the flagellation constraint and hydrodynamic considerations as discussed above. In the still environment (in which motility was important), 64-celled colonies invested more in soma, presumably to increase motility, sacrificing reproductive capacity to do so.

The among-species trend of proportion of soma increasing with cell number is paralleled within the larger (16- to 64-celled) colonies of *P. starrii*, but the high proportion of soma in the smallest (4- and 8-celled) colonies conflicts with this trend and is not predicted by the trade-off hypothesis (we discuss this issue further below). Similarly, the higher proportion of soma in 32-celled colonies from the mixed environment (compared with 32-celled colonies from the still environment) is not predicted by the trade-off hypothesis. Proportion of soma is positively but weakly correlated with the other component of size, colony diameter. This relationship is weakened by the curvilinear relationship of proportion soma with cell number, which in turn results from the high proportions of soma in the smallest (4- and 8-celled) colonies. When both colony diameter and cell number are taken into account in a statistical model that allows for non-linear relationships, they explain as much as 56% (multiple R^2) of the total genetic variance in proportion of soma. Because each isolate line is genetically homogeneous, this relationship represents a genetic correlation.

Proposed mechanism of somatic differentiation and basis for evolutionary constraint

The high proportion of somatic cells in 4- and 8-celled colonies (~50%) is far from its hypothesized optimum; comparative and hydrodynamic studies suggest that the optimal proportion of soma in such colonies is zero (Koufopanou, 1994; Solari et al., 2006). Colonies of this size are not affected by the flagellation constraint, and species with maximum cell numbers of up to 32 survive without somatic cells. Nevertheless, 4- and 8-celled colonies of *P. starrii* sacrifice half of their potential reproduction to produce somatic cells. Observed values of a critical component of fitness (reproduction) so far from its optimum suggest the involvement of a significant constraint. We now propose a mechanistic basis for such a constraint consistent with our data and the biology of *P. starrii*.

The relationship of soma to colony size, combined with the positive correlations between proportions of soma across cell number classes, suggests a possible mechanism of somatic differentiation and a basis for the hypothesized constraint. As in all *Pleodorina* species, somatic cells in *P. starrii* are located in the anterior of the colony (as defined by swimming direction). One possible explanation of this pattern is that cells differentiate in response to a threshold concentration of an anterior–posterior chemical gradient established early in development.

Cells in *P. starrii* colonies are arranged in ‘tiers’, or successive rings of cells from anterior to posterior, forming circles around the axis of rotation (Fig. 4). If a gradient/threshold system controls somatic differentiation, we should expect a preponderance of colonies to have numbers of somatic cells corresponding to whole tiers (since all cells in a tier are approximately the same distance from the anterior). This is conspicuously observed across cell number classes, with modal values of 4 somatic cells in 8-celled colonies (arranged as 2 tiers of 4 cells) and 16-celled colonies (4 tiers of 4), 12 in 32-celled colonies (4, 8, 8, 8, 4), and, in the mixed treatment, 24 in 64-celled colonies (4, 8, 12, 16, 12, 8, 4).

If direct or indirect selection on proportion of soma causes a change in either the concentration of the chemical signal or the sensitivity of cells to the signal, we would expect the proportion of soma to respond in kind across all cell number classes. The positive correlations of proportion of soma between cell number classes show that this is the case (Table 5). Cell number is fixed early in development (since cell division ends before release from the mother colony), so these correlations reflect relationships among colonies with different final numbers of cells, not trivial correlations between different developmental stages. In light of the proposed mechanism of differentiation, then, the high proportions of soma in 4- and 8-celled colonies could result from a developmental constraint that prevents independent optimization in different cell number classes. If there is selection for soma in the more common 32- and 64-celled colonies, the proposed developmental constraint may have caused a correlated response in the smaller colonies.

If a gradient/threshold mechanism controls somatic differentiation, variations from the modal numbers of somatic cells would have to be explained as developmental noise, i.e. by some but not all of the cells in a given tier responding to the chemical signal. The first tier of cells is essentially always differentiated as soma regardless of colony cell number (Fig. 4). In 16-celled colonies, one or more cells in the second tier occasionally differentiates as soma, resulting in the left-skewed distribution of somatic cells observed in these colonies (Fig. 4D, E). In 32-celled colonies, the second tier is usually differentiated as soma, but one or more cells occasionally fails to differentiate, resulting in a right-skewed distribution of somatic cells (Fig. 4H, I).

In *Volvox carteri*, somatic cell determination is controlled by the expression of the *regA* (somatic regenerator) gene, which curtails cell growth by suppressing chloroplast biogenesis (Meissner et al., 1999). *RegA* expression is in turn controlled by cell size differences established by an asymmetric division early in development (Kirk et al., 1993). It is not known if *regA* plays a role in somatic cell determination in *P. starrii*, but if it does, its expression must be controlled by something other than cell size, since all cells are the same size at the end of mitosis. The mechanism proposed here is testable, for example by experiments involving microinjection, as is the involvement of *regA*, for example by a combination of genomic data and genetic experiments.

Our results, along with the hydrodynamic studies of Solari and colleagues (Michod et al., 2006; Short et al., 2006; Solari et al., 2006), suggest a scenario for the evolution of cellular differentiation in *P. starrii*. Hydrodynamics considerations [possibly including the flagellation constraint (Koufopanou, 1994)] set an upper limit to the size a volvocine colony can attain without a loss of motility. If we assume that the undifferentiated ancestor of *P. starrii* had a similar large within-genotype variation in cell number, a selective pressure for increased size would eventually lead to a size distribution in which the largest colonies exceed this limit. If soma evolved as a way to maintain motility in these largest colonies, a constraint such as the one we propose would cause the smaller colonies to be ‘dragged along’ and develop somatic cells even if they were not needed. In other words, what we propose is a form of antagonistic pleiotropy, in which a trait – high proportion of soma – that increases fitness in large colonies comes at the cost of reduced fitness of small colonies.

The strict 1:1 relationship between fecundity and number of reproductive cells is important for this scenario: smaller colonies (in terms of cell number) automatically have lower reproductive potential, so the effects of selection are biased towards larger colonies (again in terms of cell number). In other words, for a genotype that produces a range of cell numbers, the largest colonies will produce most of the offspring. In addition, 32- and 64-celled colonies are much more common than smaller colonies, further biasing the effects of selection. Selection would therefore often favour a change (such as production of somatic cells) that increased the viability of large colonies even if it reduced the fecundity of smaller colonies.

CONCLUSION

The results of this study support the hypothesis that soma evolved in the volvocine algae to mitigate trade-offs between motility and fecundity. Benefits of motility are evident in the larger size of colonies adapted to the still environment. In the still environment, flagellar activity is an important advantage because it keeps colonies near the surface, where gas exchange with the atmosphere occurs, and possibly because it increases nutrient uptake by mixing the boundary layer (Michod et al., 2006). We expect colonies adapted to the mixed environment to invest less in flagellar activity because the spatial distribution of nutrients and dissolved gases is more homogeneous. Production of somatic cells increases motility at the expense of fecundity, and the higher proportion of soma in 64-celled colonies from the still environment reflects an increased investment in motility when it is most needed.

The trend of increasing proportion of soma from 16- to 64-celled *P. starrii* colonies is consistent with trends among species and with the trade-off hypothesis, but the high proportion of soma in 4- and 8-celled colonies requires another explanation. Genetic correlations between colony 'size' (diameter and cell number) and soma, along with the high proportion of soma in the smallest (4- and 8-celled) colonies, suggest that a developmental constraint prevents the simultaneous optimization of soma across size categories. The existence of such a constraint is further supported by the correlation of proportion of soma across cell number classes. We suggest that a mechanism in which cells respond in a threshold-dependent manner to an anterior–posterior chemical gradient would explain all of these observations. Although the specific mechanism we have proposed is speculative, either some sort of constraint or a previously unsuspected selective advantage of soma in smaller sized colonies is required to explain the sacrifice of half of these colonies' reproductive capacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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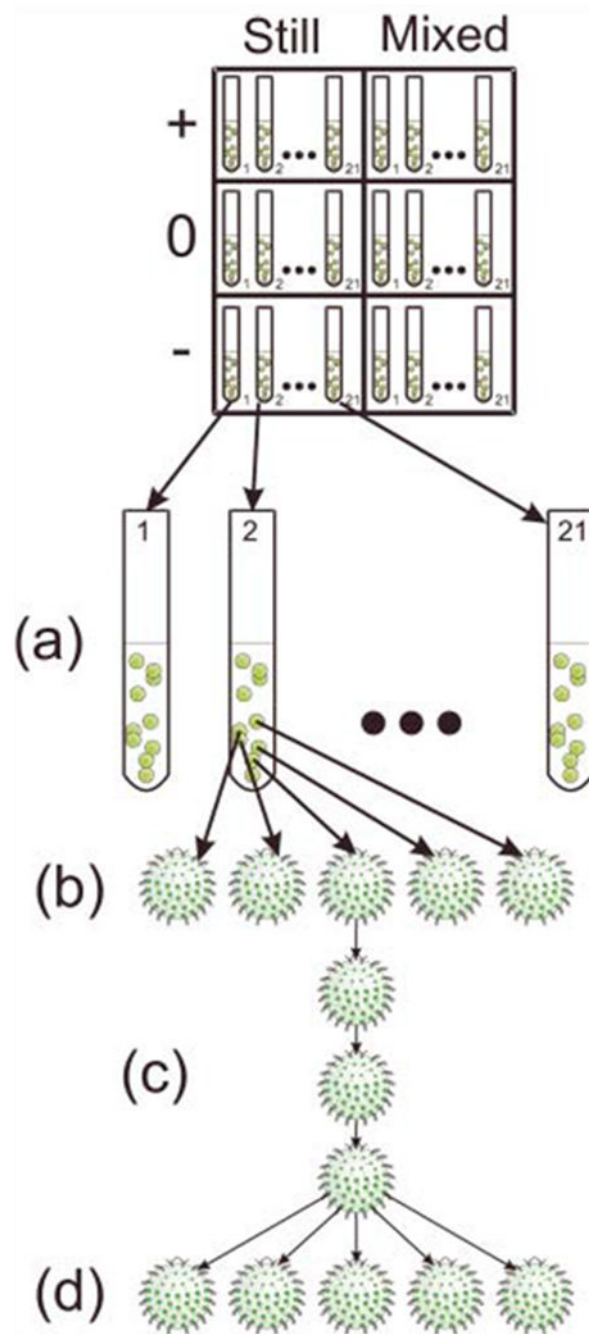


Fig. 1. Experimental design. Each combination of treatment ('+' = selection for large size, '0' = unselected control, '-' = selection for small size) and environment ('Still' and 'Mixed') was replicated with 21 populations ('tubes', a). For statistical analyses, five colonies from each tube were isolated (b) and propagated for three generations (c) in fresh, still medium to minimize environmental effects. Trait values were calculated as within-tube means from measurements on five colonies from each tube (d). Differences among colonies within tubes

(b, c, d) could include environmental and maternal effects; comparisons of means among tubes (a) are independent comparisons representing genetic differences.

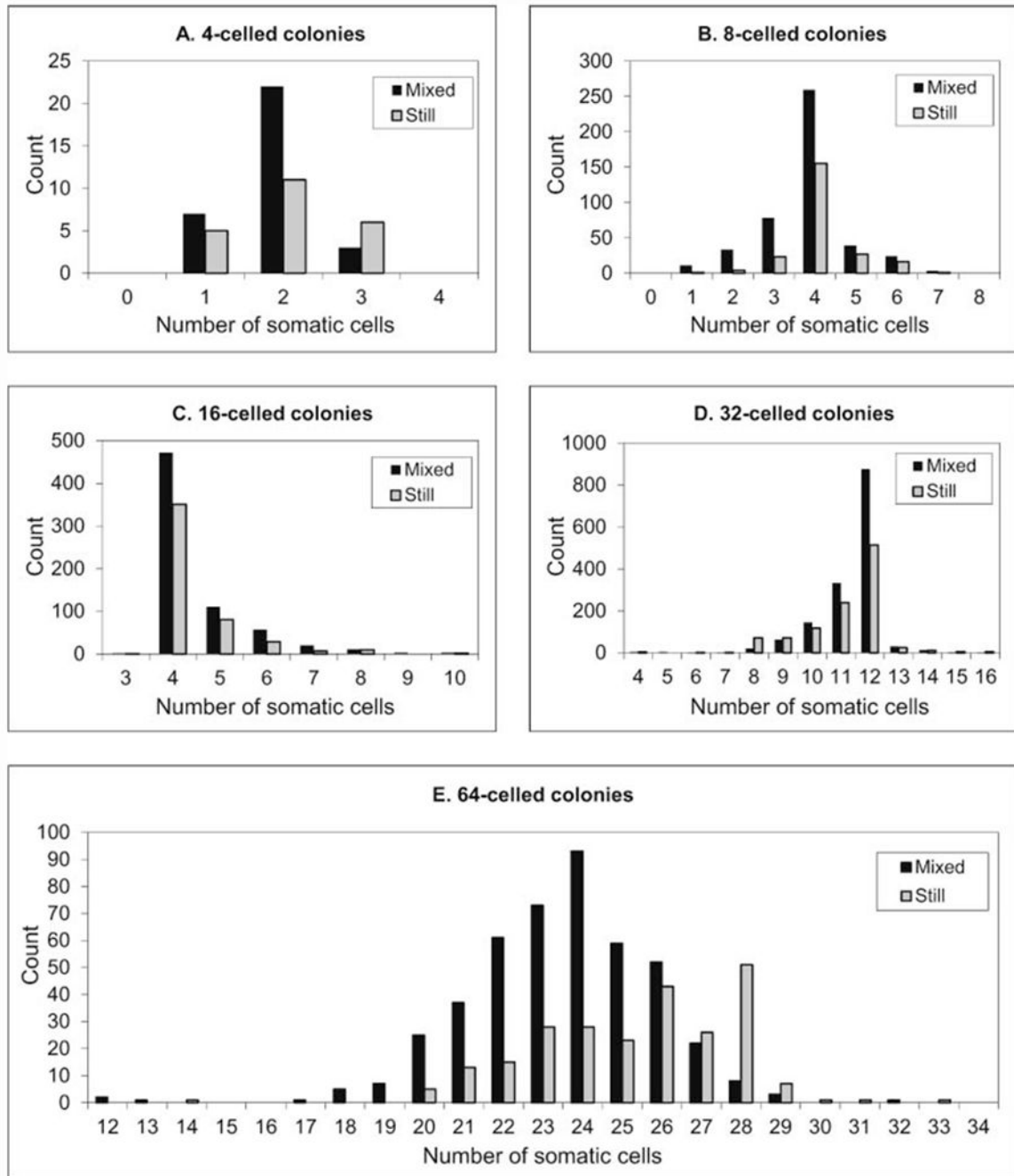


Fig. 2. Histograms of numbers of somatic cells by colony cell number. These are raw counts, not genetic values.

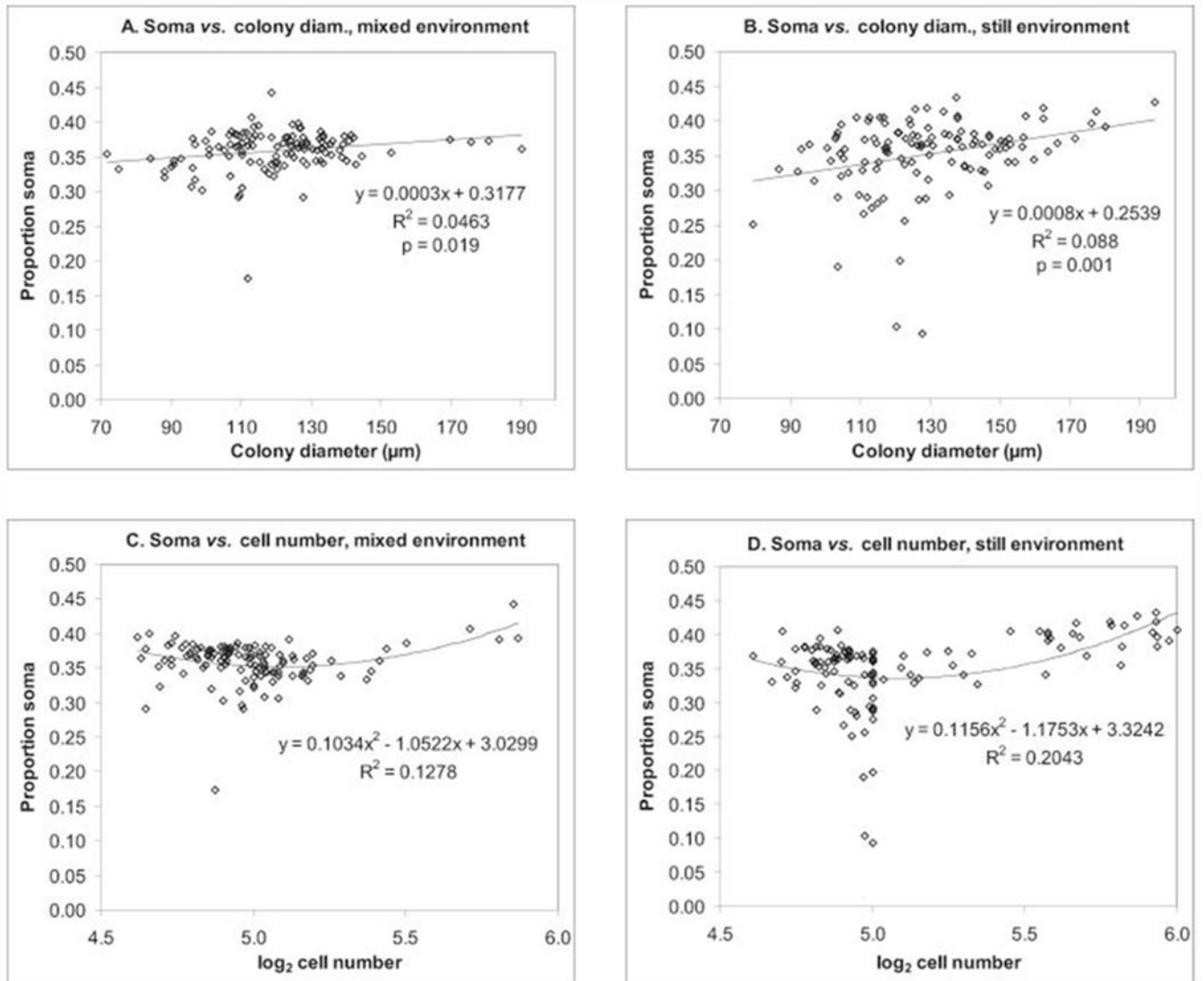


Fig. 3. Relationships of weighted average proportion soma with weighted averages of colony diameter and cell number. (A, B) linear regression; (C, D) binomial regression.

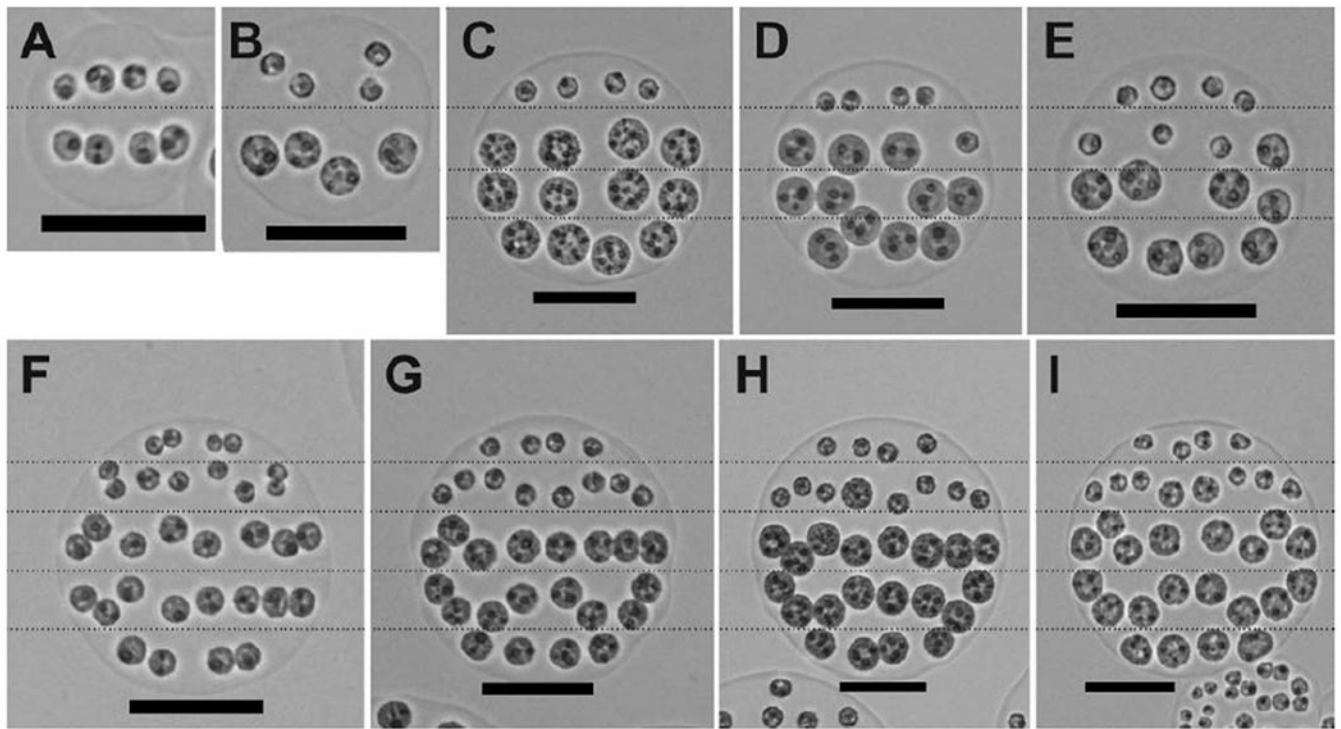


Fig. 4. *Pleodorina starii* colonies showing tiered arrangements of cells (contrast-enhanced). Photographs have been scaled to emphasize the similarities; all scale bars are 50 μm . (A) Immature 8-celled colony; (B) mature 8-celled colony; (C) mature 16-celled colony; (D) mature 16-celled colony with one cell in the second tier differentiated as soma; (E) mature 16-celled colony with three cells in the second tier differentiated as soma; (F) immature 32-celled colony; (G) mature 32-celled colony; (H) mature 32-celled colony with one reproductive cell in the second tier; (I) mature 32-celled colony with two reproductive cells in the second tier.

Table 1.Mean genetic values of colony size in isolate lines of *P. starrii* across selective treatments

Trait	Environment	Plus	S.D.	Control	S.D.	Minus	S.D.
Cell number (log ₂)	Both	5.02 ^B	0.27	4.96 ^B	0.20	5.17 ^A	0.37
Cell number (log ₂)	Still	5.06 ^B	0.36	5.02 ^B	0.22	5.25 ^A	0.46
Cell number (log ₂)	Mixed	4.99 ^{A/B}	0.18	4.90 ^B	0.18	5.09 ^A	0.28
Colony diameter	Both	127.22	21.63	124.52	20.17	121.96	18.34
Colony diameter	Still	130.29	24.38	129.46	21.54	127.25	17.24
Colony diameter	Mixed	124.14	18.47	119.58	18.71	116.66	19.37
Colony diameter (4)		64.54	17.71	64.32	8.76	63.89	7.33
Colony diameter (8)		70.87	22.93	65.78	14.53	68.40	13.18
Colony diameter (16)		90.81	15.32	84.19	14.59	85.62	16.01
Colony diameter (32)		124.23 ^{A/B}	17.58	126.91 ^A	20.09	119.19 ^B	20.63
Colony diameter (64)		149.72	32.72	150.72	39.33	141.12	27.38

Note: Numbers in parentheses are numbers of cells; traits listed without cell numbers are weighted averages across all cell number categories (see Methods). ‘Plus’ refers to selection for large size, ‘Control’ to no selection, and ‘Minus’ to selection for small size. S.D. = standard deviation of the preceding mean. Superscripts indicate significant differences among selective treatments at $\alpha = 0.05$ (pairwise comparisons using Tukey *post-hoc* test, $A > B$). Comparisons across both environments (‘Both’ in the environment column) are based on two-way ANOVAs (selective treatment + environment); all other comparisons (including all comparisons within cell number categories) are based on one-way ANOVAs (selective treatment).

Table 2.

Mean genetic values of colony size in isolate lines of *P. starrii* in still and mixed environments

Trait	Still	S.D.	Mixed	S.D.
Cell number (log ₂) [*]	5.11	0.37	4.99	0.23
Colony diameter [*]	129.00	21.27	120.13	18.85
Colony diameter (4)	69.63	17.01	60.19	10.81
Colony diameter (8) [*]	73.10	20.58	65.13	14.93
Colony diameter (16)	88.39	16.47	85.50	14.51
Colony diameter (32)	125.12	18.78	121.77	20.45
Colony diameter (64) [*]	158.59	32.59	138.53	30.59

Note: Numbers in parentheses are numbers of cells; traits listed without cell numbers are weighted averages across all cell number categories (see Methods). S.D. = standard deviation of the preceding mean.

^{*} Significant difference between means from still and mixed environments (Student's *t*-test, $\alpha = 0.05$).

Table 3.Mean genetic values of proportion soma in isolate lines of *P. starrii* across selective treatments

Cell number	Environment	Plus	S.D.	Control	S.D.	Minus	S.D.
All [#]	Both	0.37 ^A	0.03	0.34 ^B	0.06	0.36 ^{AB}	0.04
All	Still	0.36 ^A	0.04	0.33 ^B	0.07	0.37 ^A	0.04
All	Mixed	0.37 ^A	0.01	0.36 ^{AB}	0.02	0.35 ^B	0.04
4		0.52	0.11	0.56	0.24	0.56	0.08
8		0.51	0.09	0.49	0.07	0.50	0.08
16		0.30	0.07	0.30	0.07	0.28	0.04
32		0.36	0.03	0.34	0.05	0.35	0.03
64		0.38	0.03	0.36	0.04	0.37	0.04

Note: 'All' indicates weighted average across all cell number categories (see Methods). 'Plus' refers to selection for large size, 'Control' to no selection, and 'Minus' to selection for small size. S.D. = standard deviation of the preceding mean. Superscripts indicate significant differences among selective treatments at $\alpha = 0.05$ (pairwise comparisons using Tukey *post-hoc* test, $A > B$). Comparisons across both environments ('Both' in the environment column) are based on two-way ANOVAs (selective treatment + environment); all other comparisons (including all comparisons within cell number categories) are based on one-way ANOVAs (selective treatment).

[#]Significant interaction between treatment and environment.

Table 4.

Mean genetic values of proportion soma in isolate lines of *P. starrii* in still and mixed environments

Cell number	Still	S.D.	Mixed	S.D.
All [#]	0.35	0.05	0.36	0.03
4	0.55	0.16	0.52	0.12
8	0.52 [*]	0.08	0.49 [*]	0.07
16	0.29	0.07	0.29	0.06
32	0.34 [*]	0.05	0.36 [*]	0.03
64	0.39 [*]	0.04	0.36 [*]	0.03

Note: 'All' indicates weighted average across all cell number categories (see Methods). S.D. = standard deviation of the preceding mean.

^{*} Significant difference between means from still and mixed environments (Student's *t*-test, $\alpha = 0.05$).

[#] Significant interaction between treatment and environment.

Table 5.

Genetic correlations among arcsine square root proportions of somatic cells

Correlation coefficients				
Number of cells	4	8	16	32
8	0.234			
16	0.006	0.072		
32	0.210	0.066	0.198	
64	0.029	0.380	0.359	0.534
P-values (uncorrected Spearman's rank correlation)				
Number of cells	4	8	16	32
8	0.165			
16	0.790	0.153		
32	0.487	0.451	0.009	
64	0.777	0.003	< 0.001	< 0.001
P-values (uncorrected Pearson's product-moment correlation)				
Number of cells	4	8	16	32
8	0.350			
16	0.980	0.422		
32	0.314	0.414	0.007	
64	0.926	< 0.001	< 0.001	< 0.001

Note: The Bonferroni-adjusted α for a 0.05 probability of experiment-wise type I error is 0.005.