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

Deborah E. Shelton[1], Alexey G. Desnitskiy[2] and Richard E. Michod[1]

[1]*Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, USA and* [2]*Department of Embryology, St. Petersburg State University, St. Petersburg, Russia*

ABSTRACT

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

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

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

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

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

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

Correspondence: D.E. Shelton, Department of Ecology and Evolutionary Biology, University of Arizona, 1041 E. Lowell St., Tucson, AZ 85721, USA. e-mail: dshelton@email.arizona.edu

INTRODUCTION

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

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

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

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

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

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

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

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

METHODS

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

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

Table 1. *Volvox* species included in this study

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

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

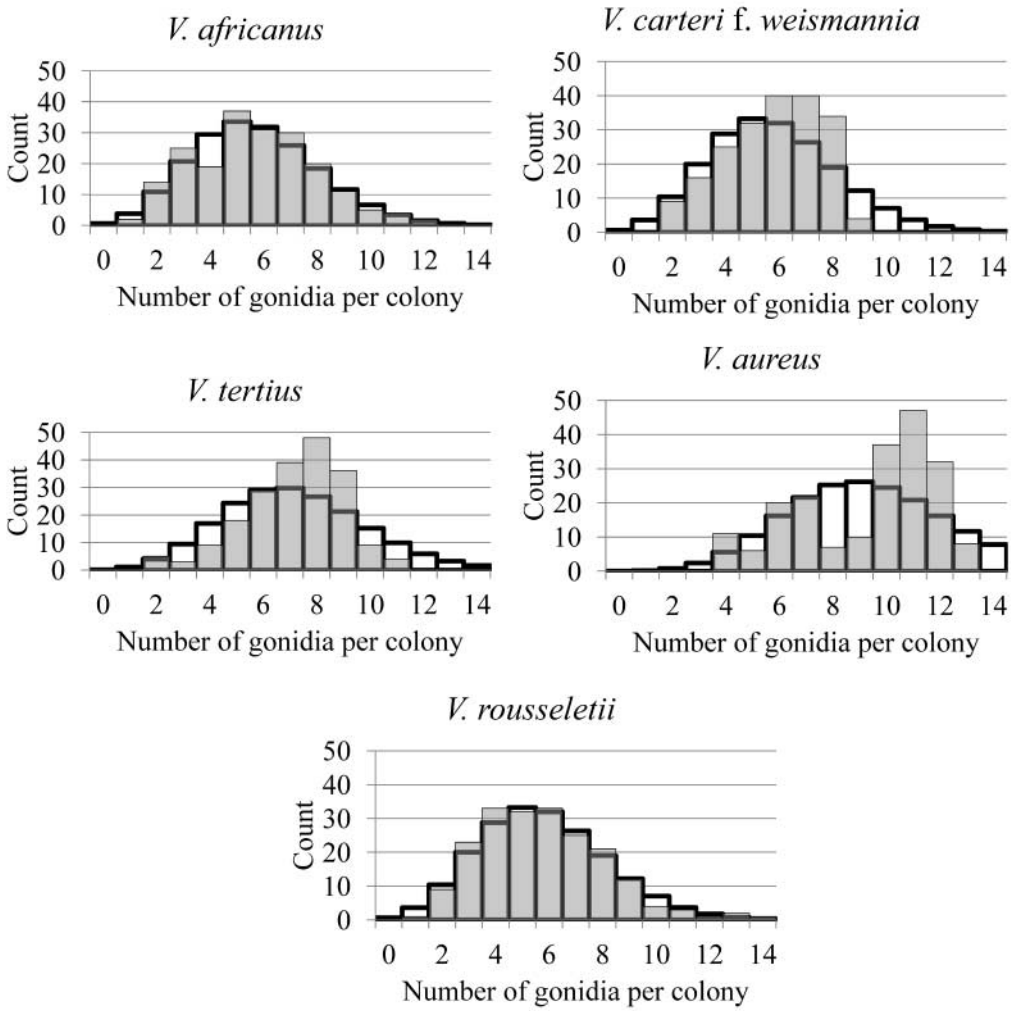


Fig. 1. Histograms showing the count of colonies that have a particular number of gonidia ($n = 200$ colonies per species). Grey bars are the observed data; white bars are the expectations based on the Poisson distribution. Maximum likelihood was used to estimate λ for each species and a chi-squared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

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

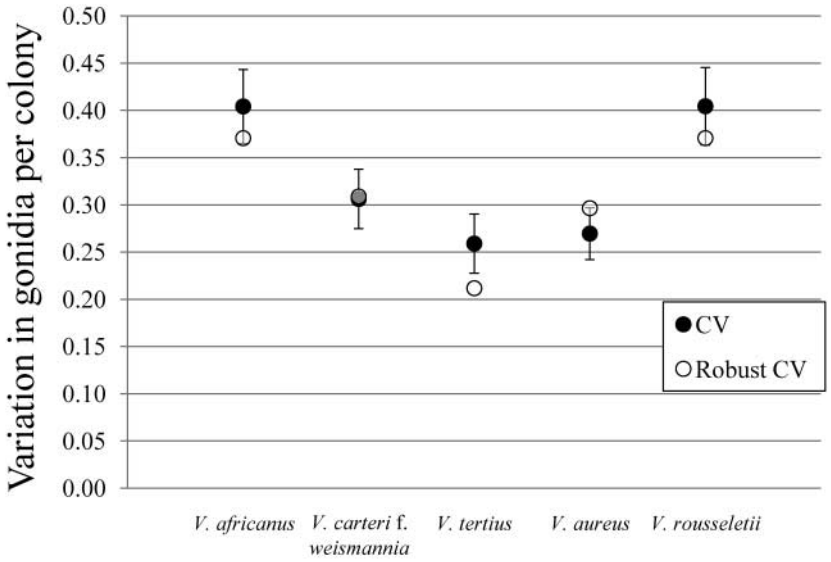


Fig. 2. Estimates of the coefficient of variation (CV) of gonidial cells per colony for each species. Black circles show the CV (sample standard deviation divided by sample mean). Error bars show ± 2 standard errors. Standard error of the CV was estimated by jackknifing (using R). Because the distributions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

MODEL

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

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

$$M_i = s_i + rg_i. \tag{1}$$

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

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

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

Table 3. Description of model parameters

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

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

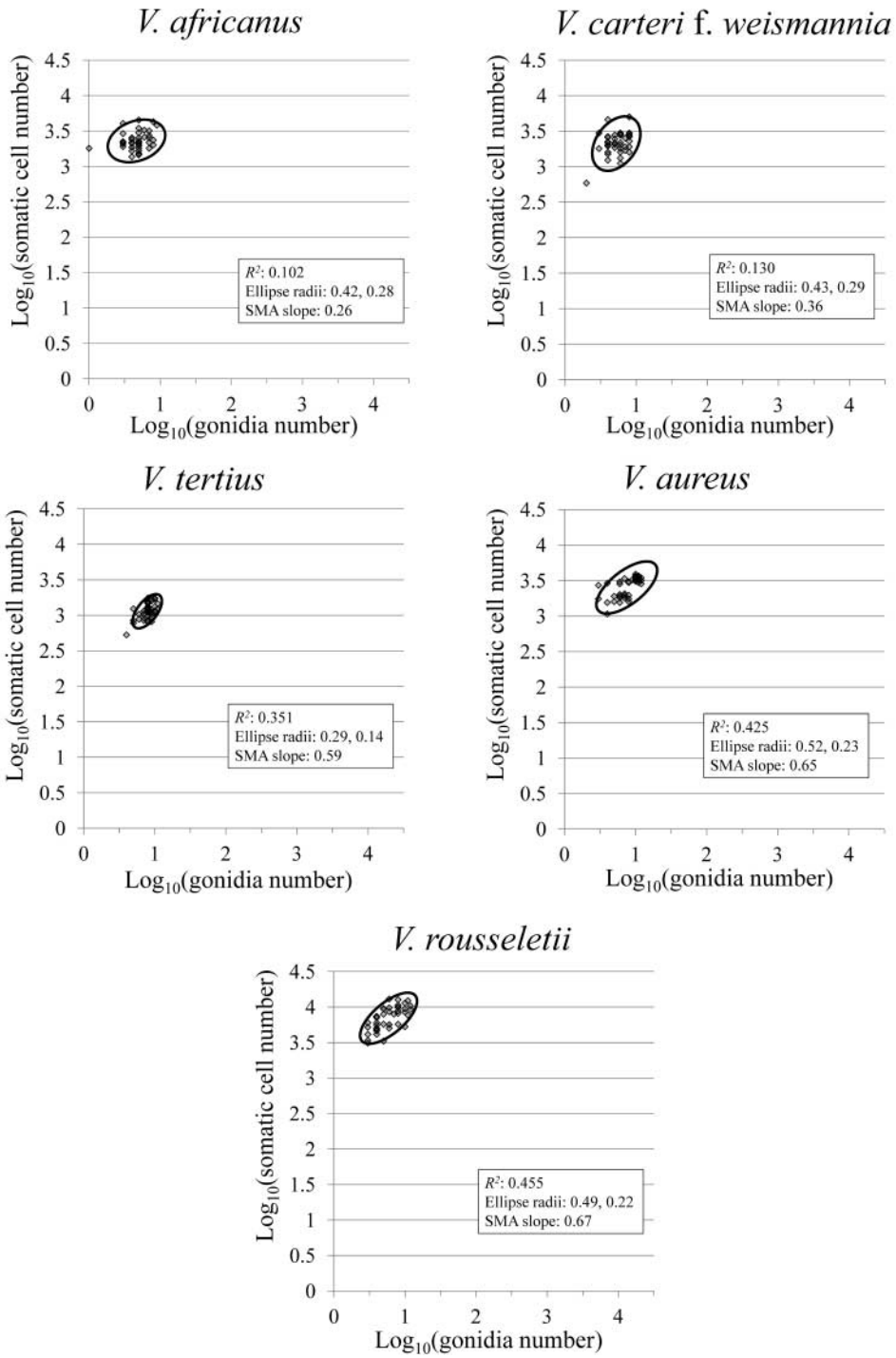


Fig. 3. Relationship between log-transformed gonidial cell number and somatic cell number for five species of *Volvox*. Each point is one colony ($n = 40$) and the ellipse is a 95% confidence ellipse. The R^2, radii of the ellipse semi-axes, and slope of a standardized major axis best-fit line are also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

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

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

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

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

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

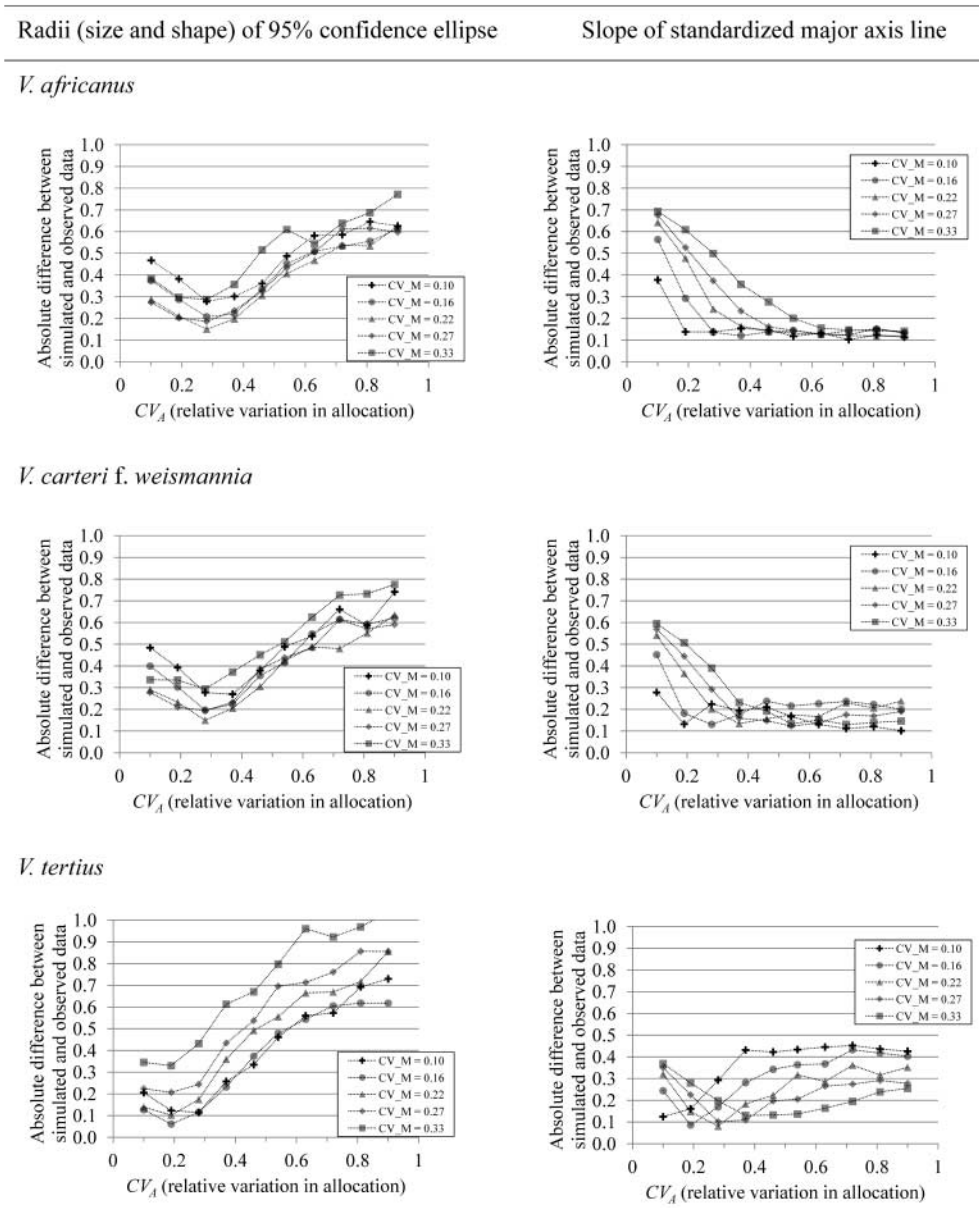
For each species, we estimated $\bar{M}$ and $\bar{A}$ from the cell count data combined with a rough estimate of r (Table 4). Although it would be ideal to have estimates of $\bar{M}$ and $\bar{A}$ that are independent of our cell count data, we do not believe this to be a major problem. Both developmental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

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

Table 4. Species-specific parameter estimates

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

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



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

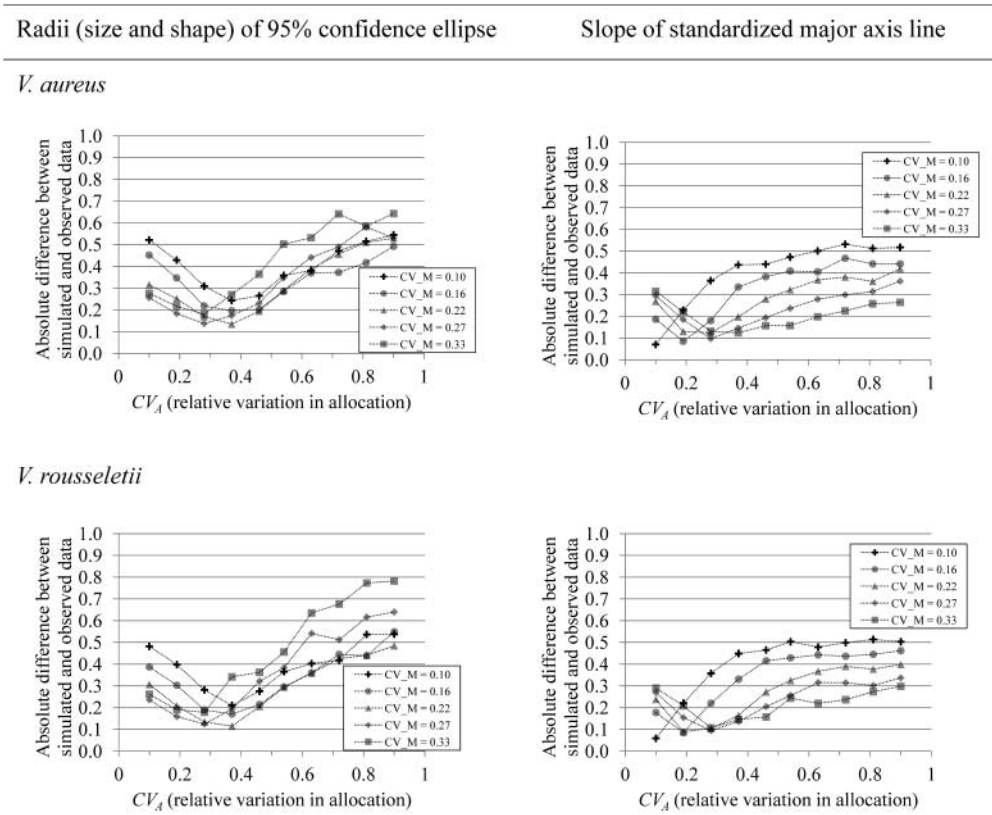


Fig. 4. The similarity of the simulated and observed data is shown according to two metrics: radii of 95% confidence ellipses (left column) and slope of SMA line (right column). Each data point represents the mean of 50 simulations with a particular combination of parameter values. The CV_A values used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

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

DISCUSSION

Non-random patterns in *Volvox* cell type distributions

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Comparisons with other organisms

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

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

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

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

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

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

Concluding remarks

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

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

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

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REFERENCES

Abell, A. 1999. Variation in clutch size and offspring size relative to environmental conditions in the lizard *Sceloporus virgatus. J. Herpetol.*, **33**: 173–180.

Azevedo, R.B. and Leroi, A.M. 2001. A power law for cells. *Proc. Natl. Acad. Sci. USA*, **98**: 5699–5704.

Blake, W.J., Kærn, M., Cantor, C.R. and Collins, J.J. 2003. Noise in eukaryotic gene expression. *Nature*, **422**: 633–637.

Brakefield, P.M. 2006. Evo-devo and constraints on selection. *Trends Ecol. Evol.*, **21**: 362–368.

Coleman, A.W. 2012. A comparative analysis of the Volvocaceae (Chlorophyta). *J. Phycol.*, **48**: 491–513.

Darden, W.H., Jr. 1966. Sexual differentiation in *Volvox aureus. J. Protozool.*, **13**: 239–255.

Desnitski, A.G. 1995. A review on the evolution of development in *Volvox* – morphological and physiological aspects. *Eur. J. Protistol.*, **31**: 241–247.

Desnitskiy, A.G. 2008. *Volvox: Evolutionary Reorganizations of Ontogeny in Related Species*. St. Petersburg, Russia: St. Petersburg University Press.

Gilbert, S.F. 2001. Ecological developmental biology: developmental biology meets the real world. *Developmental Biol.*, **233**: 1–12.

Gilles, R. and Jaenicke, L. 1982. Differentiation in *Volvox carteri*: study of pattern variation of reproductive cells. *Z. Naturforsch.*, **37**: 1023–1030.

Green, K.J. and Kirk, D.L. 1981. Cleavage patterns, cell lineages, and development of a cytoplasmic bridge system in *Volvox* embryos. *J. Cell Biol.*, **91**: 743–755.

Guinnee, M., West, S. and Little, T. 2004. Testing small clutch size models with *Daphnia. Am. Nat.*, **163**: 880–887.

Herron, M.D. and Michod, R.E. 2008. Evolution of complexity in the volvocine algae: transitions in individuality through Darwin's eye. *Evolution*, **62**: 436–451.

Herron, M.D., Desnitskiy, A.G. and Michod, R.E. 2010. Evolution of developmental programs in *Volvox* (Chlorophyta). *J. Phycol.*, **46**: 316–324.

Hill, W., Mulder, H. and Zhang, X. 2007. The quantitative genetics of phenotypic variation in animals. *Acta Agric. Scand., Section A – Anim. Sci.*, **57**: 175–182.

Hoaglin, D., Mosteller, F. and Tukey, J. 1983. *Understanding Robust and Exploratory Data Analysis*. New York: Wiley.

Huang, S. 2009. Non-genetic heterogeneity of cells in development: more than just noise. *Development*, **136**: 3853–3862.

Ireland, G.W. and Hawkins, S.E. 1981. Inversion in *Volvox tertius*: the effects of conA. *J. Cell Sci.*, **48**: 355–366.

Iyengar, M.O.P. and Desikachary, T.V. 1981. *Volvocales*. New Delhi: Indian Council of Agricultural Research.

Jacobs, M.W. and Podolsky, R.D. 2010. Variety is the spice of life histories: comparison of intraspecific variability in marine invertebrates. *Integr. Comp. Biol.*, **50**: 630–642.

Janet, C. 1912. *Le Volvox*. Limoges: Librairie Ducourtieux et Gout.

Kirk, D.L. 1998. *Volvox: Molecular-Genetic Origins of Multicellularity and Cellular Differentiation* (Developmental and Cell Biology Series). Cambridge: Cambridge University Press.

Kirk, D.L. 2001. Germ–soma differentiation in *Volvox*. *Developmental Biol.*, **238**: 213–223.

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

Kirk, M.M., Ransick, A., McRae, S.E. and Kirk, D.L. 1993. The relationship between cell size and cell fate in *Volvox carteri*. *J. Cell Biol.*, **123**: 191–208.

Kochert, G. 1968. Differentiation of reproductive cells in *Volvox carteri*. *J. Protozool.*, **15**: 438–452.

Koufopanou, V. 1994. The evolution of soma in the Volvocales. *Am. Nat.*, **143**: 907–931.

Lande, R. 1977. On comparing coefficients of variation. *Syst. Zool.*, **26**: 214–217.

Larsen, E. 2005. Developmental origins of variation. In *Variation: A Central Concept in Biology* (B. Halligrísson and B.K. Hall, eds.), pp. 113–129. Amsterdam: Elsevier.

Lewontin, R. 1966. On the measurement of relative variability. *Syst. Zool.*, **15**: 141–142.

Lynch, M. and Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sunderland, MA: Sinauer Associates.

Maynard Smith, J., Burian, R., Kauffman, S., Alberch, P., Campbell, J., Goodwin, B. *et al.* 1985. Developmental constraints and evolution: a perspective from the Mountain Lake conference on development and evolution. *Q. Rev. Biol.*, **60**: 265–287.

McCracken, M.D. and Starr, R.C. 1970. Induction and development of reproductive cells in the K-32 strains of *Volvox rousseletii*. *Arch. Protistenk.*, **112**: 262–282.

Munsky, B., Neuert, G., and van Oudenaarden, A. 2012. Using gene expression noise to understand gene regulation. *Science*, **336**: 183–187.

Newman, S.A., Forgacs, G. and Muller, G.B. 2006. Before programs: the physical origination of multicellular forms. *Int. J. Developmental Biol.*, **50**: 289–299.

Nozaki, H. 1988. Morphology, sexual reproduction and taxonomy of *Volvox carteri* f. *kawasakiensis* f. nov. (Chlorophyta) from Japan. *Phycologia*, **27**: 209–220.

Nozaki, H. and Coleman, A.W. 2011. A new species of *Volvox* sect. Merrillosphaera (Volvocaceae, Chlorophyceae) from Texas. *J. Phycol.*, **47**: 673–679.

Nozaki, H., Ott, F.D. and Coleman, A.W. 2006. Morphology, molecular phylogeny and taxonomy of two new species of *Pleodorina* (Volvoceae, Chlorophyceae). *J. Phycol.*, **42**: 1072–1080.

Ozbudak, E.M., Thattai, M., Kurtser, I., Grossman, A.D. and van Oudenaarden, A. 2002. Regulation of noise in the expression of a single gene. *Nature Genet.*, **31**: 69–73.

Pall, M.L. 1975. Mutants of *Volvox* showing premature cessation of division: evidence for a relationship between cell size and reproductive cell differentiation. In *Developmental Biology: Pattern Formation, Gene Regulation* (D. McMahon and C. F. Fox, eds.), pp. 148–156. Menlo Park, CA: W.A. Benjamin.

Pentecost, A. 1983. The distribution of daughter colonies and cell numbers in a natural population of *Vovox aureus* Ehrenb. *Ann. Bot.*, **52**: 769–776.

Pocock, M. 1933. *Volvox* in South Africa. *Ann. South Afr. Mus.*, **16**: 523–646.

R Development Core Team. 2012. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Smith, G.M. 1944. A comparative study of the species of *Volvox*. *Trans. Am. Microscop. Soc.*, **63**: 265–310.

Smith, R.J. 2009. Use and misuse of the reduced major axis for line-fitting. *Am. J. Phys. Anthrpol.*, **140**: 476–486.

Solari, C.A., Kessler, J.O. and Michod, R.E. 2006. A hydrodynamics approach to the evolution of multicellularity: flagellar motility and germ–soma differentiation in volvocalean green algae. *Am. Nat.*, **167**: 537–554.

Starr, R. 1969. Structure, reproduction and differentiation in *Volvox carteri* f. *nagariensis* Iyengar, strains HK9 & 10. *Arch. Protistenkd.*, **111**: 204–222.

Starr, R. 1970. Control of differentiation in *Volvox. Devel. Biol.*, **4** (suppl.): 59–100.

Starr, R.C. and Zeikus, J.A. 1993. UTEX – the culture collection of algae at the University of Texas at Austin 1993 list of cultures. *J. Phycol.*, **29** (suppl.): 1–106.

Sultan, S. 2007. Development in context: the timely emergence of eco-devo. *Trends Ecol. Evol.*, **22**: 575–582.

Van Noordwijk, A. and De Jong, G. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *Am. Nat.*, **128**: 137–142.

Wenzl, S. and Sumper, M. 1979. Evidence for membrane-mediated control of differentiation during embryogenesis of *Volvox carteri. FEBS Lett.*, **107**: 247–249.

Yates, I., Darley, M. and Kochert, G. 1975. Separation of cell-types in synchronized cultures of *Volvox carteri. Cytobios*, **12**: 211–223.