

The Relationship between Cell Size and Cell Fate in *Volvox carteri*

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Abstract. In *Volvox carteri* development, visibly asymmetric cleavage divisions set apart large embryonic cells that will become asexual reproductive cells (gonidia) from smaller cells that will produce terminally differentiated somatic cells. Three mechanisms have been proposed to explain how asymmetric division leads to cell specification in *Volvox*: (a) by a direct effect of cell size (or a property derived from it) on cell specification, (b) by segregation of a cytoplasmic factor resembling germ plasm into large cells, and (c) by a combined effect of differences in cytoplasmic quality and cytoplasmic quantity. In this study a variety of *V. carteri* embryos with genetically and experimentally altered patterns of development were examined in an attempt to distinguish among these hypotheses. No evidence was found for regionally specialized cytoplasm that is essential for gonidial specification. In all cases studied, cells with a diameter $> \sim 8 \mu\text{m}$ at the end of cleavage—no matter where or how these cells had been produced in the embryo—developed as gonidia. Instructive observations in this regard were obtained by three different experimental interventions. (a) When heat shock was

used to interrupt cleavage prematurely, so that presumptive somatic cells were left much larger than they normally would be at the end of cleavage, most cells differentiated as gonidia. This result was obtained both with wild-type embryos that had already divided asymmetrically (and should have segregated any cytoplasmic determinants involved in cell specification) and with embryos of a mutant that normally produces only somatic cells. (b) When individual wild-type blastomeres were isolated at the 16-cell stage, both the anterior blastomeres that normally produce two gonidia each and the posterior blastomeres that normally produce no gonidia underwent modified cleavage patterns and each produced an average of one large cell that developed as a gonidium. (c) When large cells were created microsurgically in a region of the embryo that normally makes only somatic cells, these large cells became gonidia. These data argue strongly for a central role of cell size in germ/soma specification in *Volvox carteri*, but leave open the question of how differences in cell size are actually transduced into differences in gene expression.

VISIBLY asymmetric cell division is associated with cellular differentiation in organisms as diverse as bacteria (Stragier, 1991), budding yeasts (Nasmyth and Shore, 1987), higher plants (Raven et al., 1986), and animals (Davidson, 1986). In animal embryos, visibly asymmetric cleavage divisions are frequently accompanied by segregation of demonstrably different cytoplasmic components to sister blastomeres (reviewed in Davidson, 1986). In at least some of these cases it can be demonstrated experimentally that it is the difference in cytoplasmic quality of the large and small cells produced by asymmetric division—and not their difference in size—that is the cause of their differential specification (for example see Henry, 1986). Therefore, even though there are many studies indicating that cells of many kinds do monitor their size and regulate their behavior in a size-dependent way (Fankhauser, 1945; Prescott, 1956; Johnston et al., 1977; Newport and

Kirschner, 1982; Edgar et al., 1986; Hayles and Nurse, 1986; Cossins, 1991; Henery, et al., 1992), it is frequently assumed that the visible difference in size between the products of an unequal division is probably accompanied by some invisible qualitative difference that is the actual cause of their divergent development (Horvitz and Herskowitz, 1992).

Recent evidence indicates, however, that size differences may act rather directly to trigger gene expression differences. In the bacterium *Bacillus subtilis*, for example, the first visible step in spore formation is an asymmetric division that produces a large cell destined to become a spore mother cell and a small cell destined to become a spore. After asymmetric division, a spore-specific sigma factor that was uniformly distributed before division is selectively activated in the small cell; it then initiates a cascade of differential gene expression that causes sister cells to develop entirely different phenotypes. It has now been postulated that this key

sigma factor is inactive in the predivision cell because of the unfavorable ratio of a membrane-bound activator to a cytoplasmic inhibitor, but that this ratio suddenly shifts in the direction of activation when the small cell, with its elevated membrane/cytoplasmic ratio, is produced by asymmetric division (Margolis et al., 1991). But this appears to be a rather exceptional situation; there are few if any cases in eukaryotes in which a difference in cell size has been shown to play a causal role in differential gene expression and/or dichotomous cytodifferentiation of two sister cells.

Certainly, cell size has not commonly been considered to be an important consideration in germ cell specification. Ever since the pioneering studies of Boveri (1910) and Hegner (1911), evidence has accumulated indicating that in many metazoans the formation of a germ cell lineage during early cleavage is linked to the segregation of distinctive cytoplasmic inclusions, frequently called germ plasm. In some cases, such visible cytoplasmic inclusions have been traced from the very first cleavage division through to the formation of definitive gametes (Strome and Wood, 1982). In other cases, it has been demonstrated that transfer of cytoplasm containing germ plasm can lead to ectopic development of germ cells (Ilmensee and Mahowald, 1974) or restoration of germ cells to experimentally sterilized embryos (Smith, 1966). Davidson (1986) has pointed out that in most of these cases it remains to be demonstrated that germ plasm is causally important in the initial step of germ cell specification, as opposed to being essential for some later event in germ cell differentiation. Nevertheless, it remains true that there are many cases in which it has been strongly inferred that a difference in cytoplasmic quality between sister blastomeres is causally important in differential cell specification, and few if any cases in which it has been inferred that a difference in blastomere size is the cause of differential cell specification.

Stereotyped asymmetric cleavage divisions constitute the first visible step of germ/soma differentiation in embryos of *Volvox carteri* f. *nagariensis*, a multicellular green flagellate with features that recommend it as a model system for the analysis of cellular differentiation (reviewed in Kirk and Harper, 1986 and Schmitt et al., 1992). In the sixth cleavage cycle of asexual embryos, 16 anterior cells normally divide asymmetrically to form sister cells of unequal size (Starr, 1969, 1970; Green and Kirk, 1981, 1982; Kirk et al., 1991). After 2–3 more asymmetric divisions that form additional small sister cells, each large cell stops dividing and eventually produces one new asexual reproductive cell, or 'gonidium'. The smaller cells produced by asymmetric division, plus all of the cells of the posterior hemisphere of the embryo, continue cleaving symmetrically until they have completed a total of 11 or 12 divisions; all of these cells then differentiate as biflagellate somatic cells.

The juvenile individual produced from each *V. carteri* embryo consists of ~2,000 somatic cells at the surface, and ~16 developing gonidia on the interior of a transparent spheroid. Because embryogenesis normally occurs while the gonidia (and subsequently the embryos) are held within a parental spheroid, at the end of embryogenesis the organism has a "box within a box" organization: a new generation of ~16 spheroids containing young somatic cells and gonidia are located inside a parental spheroid containing older somatic cells. About a day later, the young spheroids "hatch" from the parent and swim away to repeat the reproductive cy-

cle, while the parental somatic cells that have been left behind begin to undergo programmed death (reviewed in Kirk and Harper, 1986). Thus the two cell types produced during *Volvox* embryogenesis truly constitute a mortal soma and a potentially immortal germ line.

Although different temporal and spatial patterns of asymmetric division occur in sexually induced embryos, and in various mutants of *V. carteri*, the same relationship between cell size and cell fate is observed as in wild-type asexual embryos: large cells differentiate as germ cells while small cells become terminally differentiated somatic cells (Starr, 1969, 1970; Kirk et al., 1991). Similarly, asymmetric division followed by development of large cells as germ cells is observed in a few other species of *Volvox*, of which the best-studied has been *V. obversus* (Karn et al., 1974; Ransick, 1991, 1993).

Three different hypotheses have been proposed to account for the difference in developmental fate of the sister cells produced by asymmetric division in *V. carteri* f. *nagariensis* and its close relatives. Specifically, it has been proposed that dichotomous germ/soma specification is a consequence of (a) some property derived directly from differences in cell size at the end of cleavage (in *V. carteri* f. *nagariensis*, Pall, 1975), (b) differential distribution of a substance analogous to the germ plasm of animal embryos (in *V. carteri* f. *weismannia*, Kochert and Yates, 1970; Kochert, 1975), or (c) a difference in cell size combined with a difference in cytoplasmic quality (in *V. obversus*, Ransick, 1991, 1993).

Pall (1975) based his conclusion that "some parameter closely related to cell size is critical in determining reproductive cell versus somatic cell differentiation" in *V. carteri* f. *nagariensis* on his observations of *pcd* (premature cessation of division) mutants. Both asexual and sexual embryos of Pall's *pcd* strains ceased cleaving after only 5–7 division cycles, resulting in juveniles with far fewer—but generally much larger—cells than in wild-type juveniles. These *pcd* juveniles developed into adults with a superabundance of germ cells: whereas <1% of the cells develop as gonidia in wild-type asexual spheroids, a majority often did so in the *pcd* mutants; and whereas one half of the cells of wild-type sexual males produce sperm (Starr, 1969), virtually all cells of some sexual *pcd* males produced sperm. Based on his observed distribution of cell diameters at the end of cleavage, and the numbers of somatic and gonidial cells in adults, Pall (1975) concluded that "the minimum cell size for reproductive cell differentiation in *Volvox carteri* would be ~8 μ in diameter." The validity of this conclusion was left somewhat in doubt, however, because Pall did not trace individual cells from the end of cleavage through cytodifferentiation to determine whether, as postulated, there was a predictable relationship between the size of an individual cell at the end of cleavage and its subsequent fate. Nor was there any way to rule out the possibility that *pcd* mutations affected the germ/soma specification process directly, rather than exerting their effect indirectly through changes in cell size.

At the other end of the spectrum, Kochert and Yates (1970) concluded that germ-line specification in *V. carteri* forma *weismannia* was controlled by a germ plasm-like substance that was nonuniformly distributed in the uncleaved gonidium and the embryo derived from it. The initial observation that led to this conclusion was that individuals derived from gonidia that had been unilaterally irradiated with UV light frequently exhibited spatially nonrandom gonidial deficien-

cies. In further support of the explicit analogy that they drew to the germ plasm of animal embryos, it was subsequently reported that gonidia centrifuged before cleavage produced offspring with altered gonidial distribution patterns (Kochert, 1975). The significance of these experiments is in some doubt, however. As the authors stated in discussing the outcome of UV irradiation: "Whether the gonidia are missing on the side of the colony corresponding to that subjected to irradiation cannot be proved. . ." (Kochert and Yates, 1970). Indeed, others have inferred that it is improbable that the region of the embryo in which these gonidial deficiencies were regularly observed corresponded to the region that had received the greatest UV irradiation (Kirk and Harper, 1986). The experiments in which centrifugation of gonidia was said to cause perturbations of gonidial distribution in the next generation (Kochert, 1975) have never been described in sufficient detail to permit full evaluation.

Ransick (1991, 1993) based his synthetic view that gonidial specification in *Volvox obversus* is the result of "localized 'gonidial determinants' that only function when a certain cell volume is maintained" on the results of microsurgical modifications of embryos. In *V. obversus*, the eight large, anteriormost cells of the embryo that are produced by asymmetric division are the only cells that normally develop into gonidia (Karn et al., 1974). In Ransick's experimentally manipulated *V. obversus* embryos, neither small cells containing anterior cytoplasm, nor large cells containing only posterior cytoplasm, developed as gonidia. The only cells in experimentally manipulated *V. obversus* embryos that differentiated as gonidia were those that both inherited cytoplasm from the anteriormost part of the embryo and were above a threshold size ($\sim 8 \mu\text{m}$ in diameter) at the end of cleavage (Ransick, 1991).

Although these experimental results strongly support the conclusion that both quantitative and qualitative factors are involved in the gonidial specification process in *V. obversus*, it does not necessarily follow that this conclusion can be extrapolated to the gonidial specification process in *V. carteri* f. *nagariensis*, the organism that is more widely used for the study of cellular differentiation (Schmitt et al., 1992). In light of these uncertainties, we reexamined the gonidial specification process in *V. carteri* f. *nagariensis*, using a combination of genetic and experimental approaches. In particular, we attempted to test a prediction derived from the hypothesis of Pall (1975) that is diametrically opposed to predictions derived from the hypothesis of Kochert (1975): namely, that all cells of an asexual embryo that remain above a certain threshold size at the end of cleavage—wherever and however such cells have been produced—should develop as gonidia. The results reported here confirm this prediction. Thus, they support the conclusion of Pall (1975) that there is a relatively direct effect of cell size on the germ/soma specification process in *Volvox carteri*. But they leave open the question of how this difference in size is transduced into a difference in gene expression and cytodifferentiation.

Materials and Methods

Strains and Culture Conditions

All strains used here were derived from strains HK10 and 69-lb of *Volvox carteri* f. *nagariensis* that were obtained from the University of Texas Cul-

ture Collection of Algae (Austin, TX). In the following list each strain is identified by a mnemonic assigned at the time of isolation, followed (in parenthesis) by its access number and a brief phenotype description. EVE (W101, wild-type) is a subclone of HK10 (Harper et al., 1987); S16 (W161, temperature-sensitive cleavage abnormality), Pcd-K1 (W236, premature cessation of division), and S1s-S1 (W231, somatic cell-less) were derived from HK10 by mutagenesis with *N*-methyl-*N*-nitro-nitrosoguanidine; the derivation of G1s/Reg (W238, gonidialess/somatic regenerator) has been previously described (Tam and Kirk, 1991). Except where noted otherwise, all specimens were maintained axenically at 30–32°C in standard *Volvox* medium (SVM)¹ under illumination conditions previously described (Kirk and Kirk, 1983).

Estimation of Cell Sizes in Normal Wild-type Embryos

Published scanning electron micrographs of intact and fragmented embryos (Green and Kirk, 1981), plus unpublished micrographs from the same set of studies, were used to estimate the dimensions of cells of wild-type embryos after each of the first seven cleavage divisions. Average maximum and minimum linear dimensions along each major cellular axis were measured for 8–12 cells of like type. Where comparable measurements could be obtained from light micrographs of living embryos, these were compared with measurements made from scanning electron micrographs and found to agree within $\pm 8\%$. Because most *Volvox* blastomeres have complex shapes that change with cleavage stage (Green and Kirk, 1981), geometric formulas used to convert linear dimensions into estimated volumes were selected on the basis of the best approximation of the cell type in question (e.g., spheres, ellipsoids, etc.). For 4–64-cell embryos, a second approach was also used: measurements were made from light micrographs of heat-shocked embryos whose cells had rounded up, presumably as a result of dissolution of the cytoskeleton. Average volumes estimated from diameters of such cells agreed to within $\pm 10\%$ with volumes calculated from scanning electron micrographs as described above, and thus were averaged with the latter to produce the data reported in Table I for stages II–VI. Estimated cell volumes were then used to calculate what the diameters of the corresponding cells would have been if they were spherical; these computed figures are the values reported in Table I as "Average diameter." Cell volumes and cell diameters for stage VIII to XI embryos were then extrapolated, assuming that the total cellular volume of the embryo remained constant and that late cleavage divisions followed the stereotyped pattern previously described (Green and Kirk, 1981).

Estimation of Cell Sizes in Mutant and/or Experimentally Modified Embryos

For mutant and experimentally modified embryos the parameter of principal interest was the distribution of cell diameters after the last cleavage division. This was obtained by direct measurement of cell diameters, using video tapes or photographs taken after the embryo had inverted, the cytoplasmic bridges between neighboring cells had broken down, and the cells had taken on a spherical shape, but before the cells had begun to grow.

Monitoring of Developmental Histories

Four methods, using three different microscopes, were used to monitor developmental behaviors of various mutant or manipulated embryos. For continuous long-term monitoring and time-lapse videomicroscopy of embryos, one of two sealed culture chambers (described below) was used on an inverted Zeiss Axiomat microscope (Carl Zeiss, Inc., Thornwood, NY) that was fitted with a thermistor-regulated air curtain to maintain the temperature of the culture chamber at $32 \pm 0.5^\circ\text{C}$ (except where otherwise noted), and a low-light-level Hamamatsu video camera and Panasonic time-lapse video cassette recorder. Video recording was interrupted at intervals to record higher resolution images on photographic film; reticles functioning as a stage micrometer and ocular micrometer were also photographed to establish magnifications. Short-term or intermittent observations and photomicroscopy of living specimens were performed on a Zeiss Photomicroscope I. In certain experiments, specimens were fixed at various stages of

1. *Abbreviations used in this paper:* PEI, polyethylenimine; SVM, standard *Volvox* medium.

an experiment and were later examined on a Wild microscope with built-in camera lucida and used to generate detailed drawings. This had the advantage of permitting cells in all focal planes of a large, complex individual, to be counted, measured, and evaluated with respect to phenotype.

Method 1. A single individual was monitored continuously through one complete life cycle, from the time it began to cleave until its descendant gonidia began to cleave 2–3 d later. For this purpose, mature gonidia were placed on sterile cover slips in microdrops, overlaid with an agar-gelatin film, and enclosed in a custom-built culture chamber that permitted a continuous, slow flow of sterile culture medium (J. D. Pickett-Heaps, personal communication). At the conclusion of the recording, video tapes and photomicrographs were used to identify individual cells that had developed either as somatic cells or as gonidia, and to trace those cells backward in time and determine their sizes at the end of embryogenesis. This method permitted detailed analysis of the developmental history of individual cells. However, it had two disadvantages: it permitted the monitoring of only a single embryo every 2–3 d, and it was only suitable for strains that remained immobile throughout development because they produced too few somatic cells to swim (strains Pcd-K1 and Sls-S1, plus heat-shocked embryos of the Gls/Reg strain). Even when motile specimens could be successfully held within the photographic field by the agar-gelatin overlay, they usually rotated enough to preclude continuous recognition of individual cells in the postembryonic period.

Method 2. Larger numbers of embryos were monitored by time-lapse videomicroscopy at an intermediate level of resolution for a shorter time by placing 15–25 embryos at selected cleavage stages in a close-packed pattern on the lower cover glass of a Sykes-Moore culture chamber (Bellco Biotechnology, Vineland, NJ). These cover glasses had been pretreated with 0.01% polyethylenimine (PEI; Sigma Chemical Co., St. Louis, MO), which formed a bond to the glycoprotein vesicle surrounding each embryo that was just strong enough to hold the embryos in place throughout cleavage and inversion, but weak enough to permit their later removal with a micropipette. Once the video recording was begun, a mylar sheet was placed over the face of the video monitor and all embryos in the chamber were traced and given identifying numbers. After the completion of embryogenesis, and before the juveniles could hatch from their vesicles and swim away, recording was terminated, the chamber was opened, and the embryos (individually identified from the mylar tracing) were removed with a micropipette and transferred to individual wells of multi-well plastic culture dishes. After cytodifferentiation, these specimens were then individually transferred to microscope slides and observed and photographed. The number of somatic cells and gonidia in each of these specimens could then be related to numbers and sizes of cells present in the corresponding embryo at the end of cleavage, even though it was not possible to derive one-to-one identifications of individual cells. For monitoring the cleavage behavior and subsequent development of isolated blastomeres (described below) method 2 was modified by precoating cover slips with a thin film of agarose instead of PEI, since vesicle-free cells are killed by contact with PEI or glass.

Method 3. Embryos that had been subjected to cytoplasmic ligation (described below) were cultured throughout embryogenesis in micro-wells <1 mm in diameter and ~2 mm deep that had been formed in a 3-mm-deep layer of 1% SeaPlaque agarose (FCM BioProducts, Rockland, ME) in 30-mm plastic petri dishes. These micro wells were far from ideal optically, but they did permit cleavage behavior to be monitored microscopically while protecting the embryo from mechanical disturbances that otherwise would have caused the ligated lobe to detach from the embryo. Once these operated embryos had inverted and become mechanically more stable, they were transferred to conventional multi-well plastic culture dishes and monitored for the next several days as in method 2.

Method 4. At the opposite extreme from method 1, up to 500 embryos of a given type and initial cleavage stage were maintained in screw-cap culture tubes from which samples were withdrawn at intervals for analysis. Method 4 did not allow conclusions to be drawn about the developmental histories of individual cells, but it did permit correlations to be made between the cell sizes and cell types present within relatively large samples of the cultured population at various stages of development. Method 4 was modified for monitoring the development of isolated blastomeres by use of agarose-coated culture dishes.

Heat Shock

Heat shock conditions were similar to those previously reported (Kirk and Kirk, 1985, 1986), namely: 40 min at 42.5°C followed by 20 min at 45°C. Embryos to be treated were mechanically released from parental spheroids and sorted by cleavage stage, using a micropipette and dissection microscope. Bulk heat shock experiments were performed on samples of 100 or

more embryos of a single stage that were placed in tubes of liquid medium, heat shocked in water baths and then monitored by method 4. Additional heat shock experiments were performed on smaller numbers of embryos that were under long-term microscopic observation and time-lapse videomicroscopy (methods 1 or 2, above). Transients between desired microscope-stage temperatures were achieved within 15–30 s by use of an auxiliary hair drier to provide rapid heating or cooling of the microscope stage and culture chamber; temperatures at the new set point were then maintained within $\pm 0.5^\circ\text{C}$ by the air curtain.

Microsurgery

Embryos were isolated and microsurgical manipulations were performed using previously described methods (Ransick, 1991). In preparation for blastomere isolation, mature gonidia were removed from their glycoprotein vesicles microsurgically. Eight-cell embryos were first dissected into individual cells; then their daughter cells were separated from one another after the next division. In most experiments, care was taken to control the anterior-posterior orientation of the blastomeres at the first stage of separation so that at the fourth division the eight anterior blastomeres could be unambiguously distinguished from their posterior sister cells. The progeny of the isolated blastomeres were then observed and photographed at intervals over the next two days by method 2 or 4.

The ligation method previously described for *V. obversus* (Ransick, 1991) was used to produce large cells in regions of the *V. carteri* embryo that normally produce only small somatic cells. Briefly, the method was as follows: just before cleavage, the surrounding vesicle was punctured near the anterior end of a gonidium. Then pressure was applied to the posterior region of the vesicle so that most of the gonidium (including the nucleus, which is always located at the extreme anterior of the gonidium at this stage) was expelled, but a lobe of nucleus-free cytoplasm was retained within the vesicle, connected to the remainder of the gonidium by a constricted cytoplasmic stalk. After 4–6 divisions of the nucleated part of the embryo, pressure was once again applied to the vesicle, to expel the cytoplasmic lobe. In successful cases, this lobe now fused with the cell it was attached to, and thereby formed an experimentally enlarged cell that participated in subsequent cleavage and differentiation events (see Fig. 9). In all cases, ligated embryos were sketched or photographed in early cleavage to record the location of the ligated lobe with respect to the A-P axis of the embryo.

Controls for blastomere isolations and cytoplasmic ligations included: embryos from the same culture that were left in intact parental spheroids, isolated embryos left within their vesicles, and isolated embryos that were removed from their vesicles but left otherwise unaltered.

Scoring of Cell Types

Cellular phenotypes were scored 24 h after the cessation of cleavage. Cells with flagella and eyespots were scored as somatic cells, and cells with large vacuoles and central nuclei with prominent nucleoli were scored as gonidia. In all cases studied (except for the Gls/Reg mutant, in which cells normally have the somatic phenotype at day one but have begun to redifferentiate as gonidia by day two) these features were perfectly correlated with subsequent developmental behavior: cells scored as gonidia at day one went on to cleave and produce progeny, whereas those that had the somatic phenotype at day one did not.

Results

The goal of the present study was to distinguish among three alternative hypotheses regarding germ cell specification in *V. carteri*, namely, the hypotheses that such specification is a consequence of (a) quantitative, (b) qualitative, or (c) a combination of quantitative and qualitative differences in the cytoplasm of cells at the end of cleavage (Pall, 1975; Kochert, 1975; Ransick, 1991). Because there was no obvious way to modify cytoplasmic quality in a way that would necessarily be relevant, we took several different approaches to modify the distribution of cell sizes present at the end of cleavage as a way of testing the differing predictions made by these three hypotheses. However, it appeared obvious that information about the sizes of prospective germ and somatic

cells at various stages of development in control, wild-type *V. carteri* embryos should contribute to the interpretation of the outcome of experiments in which cell size was altered.

Cell Sizes in Normal Wild-type Embryos

The volumes and diameters of cells of normal, wild-type embryos at each stage of cleavage were estimated as described in Materials and Methods. These data (Table I) should be taken as approximations only, but they provide a useful context in which to view the results of other aspects of the present study. A nearly 50% reduction in volume occurs before the first cleavage division as the gonidium condenses (by emptying of vacuoles) and flattens on its anterior and posterior surfaces (Starr, 1969, 1970; Green and Kirk, 1981; also see Fig. 1 A). However, the total cellular volume of the embryo apparently remains relatively constant through the remainder of cleavage (Table I). Estimated cell volumes at stage VI indicate that after the first asymmetric division the larger cell (a gonidial initial) is only about twice the volume of its smaller sister cell (a somatic initial). But through the combined effects of three successive asymmetric divisions and fewer total divisions (9 vs. 11), at the end of cleavage the presumptive gonidia normally have a volume ~ 30 -fold greater than that of presumptive somatic cells.

Table I. Estimated Sizes of Cells at Each Stage of Cleavage in Wild-type *V. carteri* Embryos

Cleavage stage	Average diameter	Volume/cell	Total volume	Percent of gonidial volume
	μm^*	$\mu\text{m}^3\ddagger$	$\mu\text{m}^3§$	
Uncleaved gonidium	55	87,100	87,100	100
I (2-cell)	36	25,700	51,400	59
II (4-cell)	28	11,500	46,000	53
III (8-cell)	23	6,300	50,400	58
IV (16-cell)	18	3,050	48,900	56
V (32-cell)	14	1,420	45,300	52
VI (64-cell)	G 13	1,050	16,800	54
	S 11	630¶	30,200	
VII (128-cell)	G 12	820	13,100	56
	S 8	320	35,800	
VIII (256-cell)†	G 11	740	11,800	55
	S 6.5	150	36,100	
IX (512-cell)†	G 10.5	625	10,000	55
	S 5.2	77	38,000	
X (1008-cell)†	G 10.5	625	10,000	55
	S 4.2	38	38,000	
XI (2000-cell)†	G 10.5	625	10,000	55
	S 3.3	19	38,000	

* Gonidial diameter by direct measurement, other diameters calculated from estimated volumes, assuming spherical cells. After stage V, G = gonidial initials; S = somatic initials.

† Estimated as described in Materials and Methods.

‡ Estimated on the assumption that the number of cells of each type present at each stage was that predicted by the analysis of Green and Kirk (1981).

§ Average of two cohorts of somatic-initial cells: 16 small anterior cells with a mean volume of $450 \mu\text{m}^3$ (diameter $\sim 9.6 \mu\text{m}$) and 32 posterior cells with a mean volume of $720 \mu\text{m}^3$ (diameter $\sim 11.2 \mu\text{m}$). Such size differences persist throughout the remainder of cleavage and subsequent development.

¶ Extrapolated from data at earlier stages, assuming constant embryonic volume and division of all somatic initials in each cycle.

Cell Size vs. Cell Fate in Embryos of Mutants

Mutants Exhibiting Premature Cessation of Division. Pall (1975) concluded from his study of *pcd* mutants that cell size plays a crucial role in germ-soma specification in *V. carteri* f. *nagariensis*. However, he did not follow individual cells throughout development to determine whether the postulated relationship between cell size at the end of cleavage and subsequent cell fate was a direct one. Because Pall's *pcd* mutants are no longer available, we attempted to isolate strains of similar phenotype to perform such cell-by-cell analysis of development. The two strains we isolated and used (Pcd-K1 and Sls-S1) resemble Pall's strains in that they initiate cleavage in a normal pattern (Fig. 1, A and C), but then cease cleaving prematurely. However, these two strains differ from each other and from the strains that Pall described in the details of cleavage arrest. Pall concluded from the discontinuous distribution of cell numbers in his mutant adults (30–32, 60–64, or 121–128 cells per individual) that all cells of a given embryo tended to divide about the same number of times (5, 6, or 7, as opposed to the usual 11). In contrast, cells of our Pcd-K1 and Sls-S1 mutants appear to withdraw from the cleavage program individually and stochastically after 3–10 divisions, resulting in a population of juveniles with a rather continuous distribution of cell numbers and cell sizes (Fig. 1, B and D). On average, Sls-S1 cells divide ~ 5 times, whereas Pcd-K1 cells divide ~ 6 times, but otherwise the two strains are rather similar in their developmental behaviors. (The smaller number of cleavage divisions in the Sls-S1 strain led us to conclude erroneously, at the time it was isolated and named, that this mutant fails to make any somatic cells; it actually does generate a small number of somatic cells in many cases.)

Mature gonidia of the Pcd-K1 and Sls-S1 strains were isolated and followed by time-lapse videomicroscopy and photomicroscopy through one complete asexual life cycle, from the time they began to cleave until their progeny gonidia began to cleave ~ 2 d later. Video tapes and photographs were then used to follow as many individual cells as possible through the entire cycle and to relate the diameter of each of these cells at the end of cleavage to its differentiated phenotype. As a rule, $\sim 1/3$ – $1/2$ of the cells in an individual could be traced in this manner. Because the two strains produced similar results, data from the two strains were pooled. In both of these mutant embryos all cells with a diameter of $>9 \mu\text{m}$ at the end of embryogenesis developed as gonidia, all cells with a diameter of $<6 \mu\text{m}$ developed as somatic cells, and cells between 6 and $9 \mu\text{m}$ in diameter exhibited an increasing tendency to develop as gonidia with increasing size (Fig. 2).

A Mutant with a Temperature-sensitive Cleavage Defect. Strain S16 exhibits a temperature-sensitive defect of the cytokinetic apparatus such that cleavage planes become increasingly randomized with increasing temperature in the range of 24 – 37°C (a range over which wild-type embryos develop normally). Ultrastructural analysis indicates that these cleavage defects are associated with gross morphological abnormalities of the basal body complex and its associations with the plasmalemma and the nucleus (Mattson, 1984). This strain defines the locus *cleA*, and has been assigned the genotype *cleA*^{ts-1} (Kirk et al., 1991).

The motility of S16 juveniles precluded using method 1 to

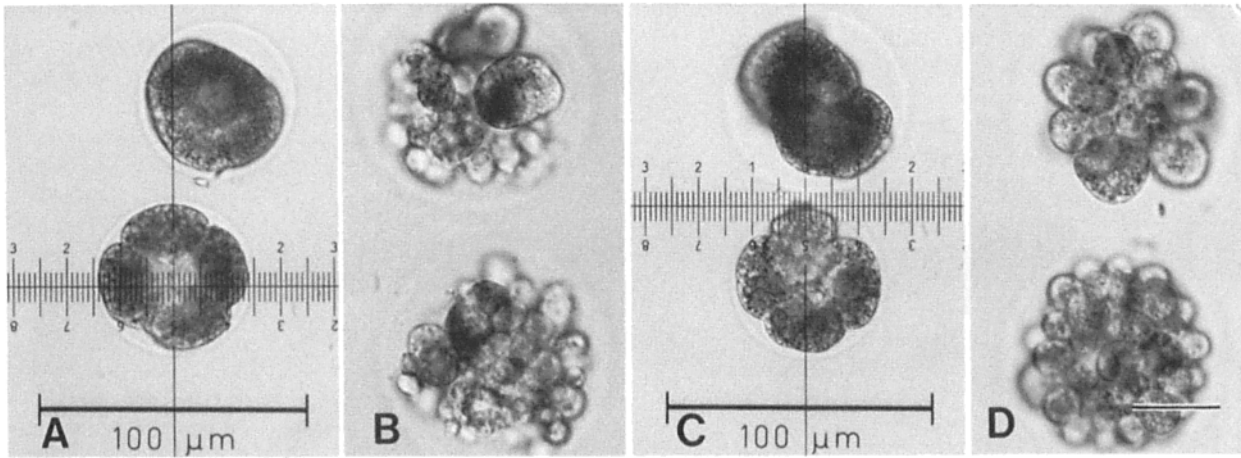


Figure 1. Cleavage in representative Pcd-K1 (*A* and *B*) and Sls-S1 (*C* and *D*) embryos. In *A*, one embryo is at the 4-cell stage and the other is condensing at the anterior pole in preparation for cleavage. In *C*, both embryos are at the 4-cell stage, but one is viewed from the anterior pole and the other is viewed from the side. Note that although cleavage of these embryos appears quite normal at early stages, by the end of cleavage (*B* and *D*) an extremely broad assortment of cell sizes has been produced because individual blastomeres ceased cleaving prematurely, but at different stages. In *A* and *C*, the reticles used for cell measurements (the 100- μm bar functioning as a stage micrometer and the graduated cross hair functioning as an ocular micrometer) were included in the photograph. Bars: (*A* and *C*) 100 μm ; (*B* and *D*) 25 μm .

monitor individual cells throughout the life cycle, and the dispersion of cell sizes produced by S16 at restrictive temperatures precluded using method 2 to establish meaningful relationships between cell size and subsequent phenotype for individual embryos. Therefore, S16 development was monitored by method 4. Samples containing ~ 250 mature gonidia from an S16 culture that had been grown at 24°C

were incubated at 24, 32, or 37°C during cleavage and then returned to 24°C. As previously observed (Mattson, 1984), S16 embryos that cleaved at 24°C generated a superficially normal adult phenotype (Fig. 3 *B*). However, closer analysis revealed that even at this "permissive" temperature S16 embryos cleaved fewer times and produced more gonidia and fewer somatic cells than wild-type embryos do (Figs. 3 *B* and 4). These abnormalities increased in severity with increasing temperature (Figs. 3, *C* and *D* and 4). Although asymmetric divisions occurred without any discernible spatial or temporal regularity in S16 embryos at elevated temperatures, resulting in a random array of cells of various size classes throughout the embryo, the number of cells with diameters $>8 \mu\text{m}$ at the end of embryogenesis was strongly correlated with the number of viable gonidia that were eventually produced (Fig. 4). This result was consistent with the hypothesis that all viable cells $>8 \mu\text{m}$ —whenever and wherever they happened to be generated during the erratic cleavage program of an S16 embryo—developed as gonidia.

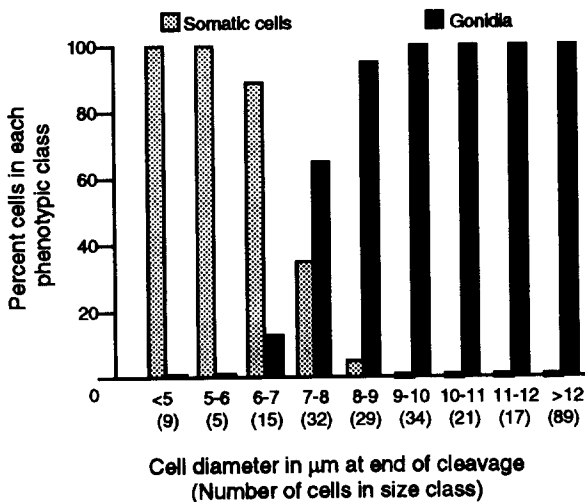


Figure 2. Differentiated phenotype as a function of cell diameter at the end of cleavage in Pcd-K1 and Sls-S1 mutants. Five individuals of each strain were monitored by method 1 through one full asexual life cycle. A total of 251 individual cells that could be traced unambiguously throughout this period (\sim half of all cells present in these specimens) were analyzed with respect to their diameters at the conclusion of embryogenesis and their differentiated phenotypes 2–3 d later. Because the results obtained with the two mutant strains exhibited extremely similar trends, the data were pooled.

Cell Size vs. Cell Fate After Interruption of Cleavage by Heat Shock

Wild-type Embryos. In a preliminary search for ways to cause a premature cessation of cleavage without blocking postembryonic development, many metabolic inhibitors (such as aphidocolin, actinomycin D, anisomycin, and chloramphenicol) and cytoskeletal inhibitors (such as cytochalasin and nocodazole) were tested at various concentrations and stages of embryogenesis. All of these drugs had one of two effects: either they did not interrupt cleavage, or they also blocked subsequent development irreversibly. Cold shocks of varying intensity and duration had similar effects. However, when heat shock (42.5°C for 40 min followed by 45°C for 20 min; Kirk and Kirk, 1985, 1986) was applied to embryos at the 32-cell stage or beyond, it caused a truncation of the cleavage program without causing any significant amount of cell death or blocking subsequent development.

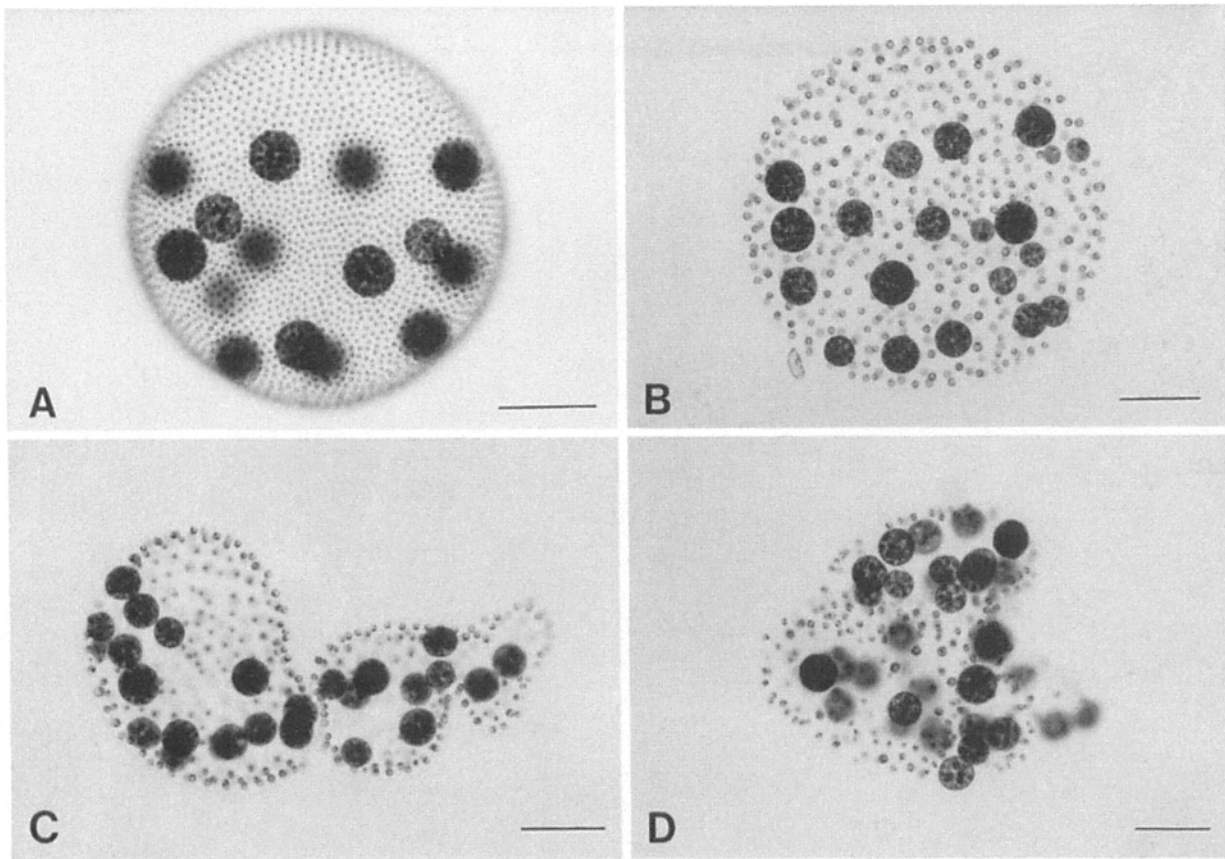


Figure 3. Representative phenotypes of S16 adults produced from embryos that were maintained at different temperatures during cleavage. A control spheroid produced from a wild-type embryo that cleaved at 37°C (A) is provided for comparison with S16 individuals produced from embryos that cleaved at 24 (B), 32 (C) and 37 (D)°C. Note that although the morphology of the spheroid in B appears superficially to be quite normal, relative to the wild-type spheroid it has more gonidia (of more variable size) and fewer somatic cells; its overall organization is also less regular. These abnormalities increase with increasing temperature during cleavage. The multi-lobed appearance of many S16 individuals (as in C) is a result of inversion abnormalities that are secondary to cleavage abnormalities (Mattson, 1984). Bars, 100 μm.

In preliminary experiments in which 32–128-cell embryos were heat shocked and monitored in bulk (method 4) it was determined that the number of divisions subsequently completed was variable both within and among individuals heat

shocked at the same stage, but often averaged between one or two, and only rarely averaged more than three. Adults derived from cultures heat shocked at the 32–64-cell stage frequently had fewer than 128 cells, and in most such cases a majority of these cells developed as gonidia that cleaved within 2 d. In one particularly striking case, an individual heat shocked at the 64-cell stage produced 124 gonidia and only eight somatic cells.

More detailed studies were performed by continuous video and photographic monitoring of 32–128-cell embryos (by method 2) before, during and after heat shock. At all cleavage stages, heat-shocked cells quickly rounded up and ceased dividing (Fig. 5, A and B), presumably reflecting a partial disruption of the cytoskeleton. In the rare cases in which cells were observed to divide during heat shock, division always occurred in the first few minutes, indicating that it was probably already underway before the temperature was raised. As in the preliminary experiments, the number of divisions that occurred after heat shock was variable both within and among embryos of similar stage: some cells never divided again, some divided one to three more times, and only a small minority completed as many as four additional divisions. Many of these embryos produced an extreme superabundance of gonidia and only a few somatic cells (Fig. 5, C and D).

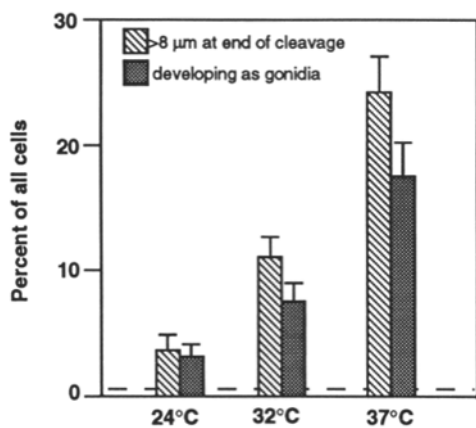


Figure 4. The mean abundance of cells >8 μm at the end of cleavage (▨) and of cells developing as gonidia (■) in S16 individuals held at different temperatures during cleavage. Error bars indicate standard deviations.

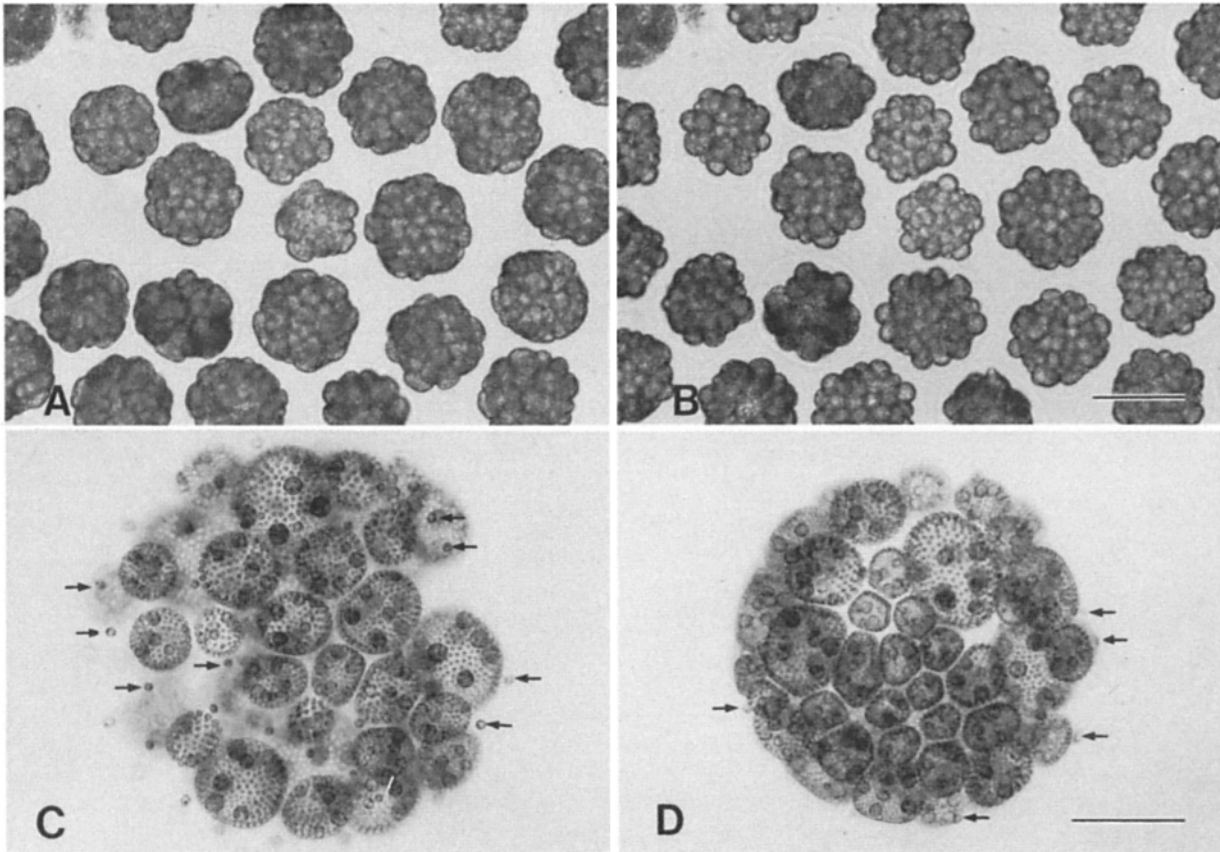


Figure 5. The effects of heat shock on cleaving wild-type embryos. (A) A collection of mid-cleavage embryos being monitored by method 2; photographed just before the initiation of heat shock. (B) The same set of embryos photographed immediately after the termination of heat shock. Note that most embryos have failed to divide in the intervening hour, and most cells remain rounded up as a consequence of the heat shock. (C) An adult spheroid derived from an embryo that had been heat shocked two days earlier at the 64-cell stage. This individual produced 48 gonidia (that by this time had enlarged and cleaved to produce juvenile second-generation spheroids) and 65 somatic cells (arrows). (D) A similar individual in which 55 gonidia (now juvenile spheroids) and only 19 somatic cells (arrows) were produced. Bars: (A and B) 50 μm ; (C and D) 100 μm .

Nearly 160 embryos that had been heat shocked at the 32–64-cell stage while being monitored by method 2 were analyzed at the end of inversion to determine cell diameters, and then again 2 d later to determine the numbers of total cells, somatic cells and gonidia present. At 2 d after heat shock, >98% of all cells could be unambiguously identified as either somatic cells or gonidia, even though both cell types exhibited somewhat more size variation than cells of control spheroids. Although all of these individuals had been subjected to the same environmental stress, they exhibited a clear, inverse relationship between the total number of cells that had been produced by the time division stopped and the proportion of those cells that differentiated as gonidia (Fig. 6; Table II). More than half (88) of these individuals possessed more gonidia than somatic cells. Among 63 individuals that contained 90 cells or fewer, the number of gonidia per spheroid ranged from 26 to 74, with a mean of 45 (SD = ± 14). In marked contrast, the 16 individuals that contained 128–256 cells, had a mean of 22 (± 9) gonidia, and the five individuals that produced more than 256 cells had the normal 16 (± 1) gonidia.

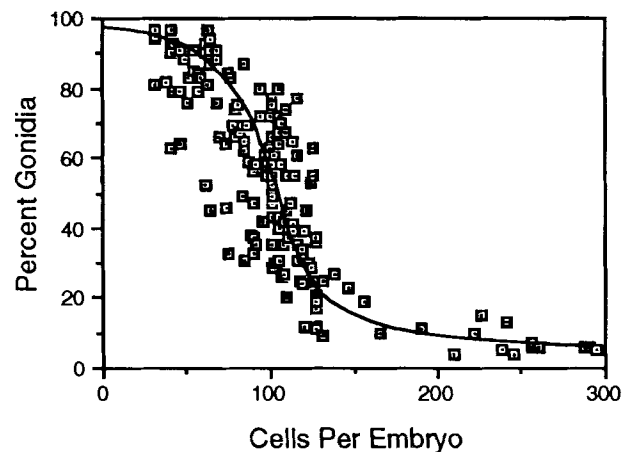


Figure 6. Relative abundance of gonidia, as a function of total cell number, in 158 spheroids that were derived from embryos that had been heat shocked at the 32- and 64-cell stages while being monitored by method 2. The line was fitted by eye, merely to indicate the general trend of the data

Table II. Gonidial Abundance in Heat-shocked Wild-type Embryos as a Function of Cell Number

Cell number (number of embryos)	Number of gonidia*	Percent gonidia*	Average diameter of small cells $\mu\text{m}^{\dagger\dagger}$
32–64 (34)	42 ± 12	85 ± 12	11.2 ± 4.1
65–90 (29)	50 ± 13	62 ± 18	8.4 ± 2.6
91–110 (43)	55 ± 17	54 ± 17	7.7 ± 1.8
111–128 (31)	44 ± 14	36 ± 13	8.1 ± 1.9
129–256 (16)	22 ± 10	12 ± 8	7.2 ± 1.3
>256 (5)	16 ± 1	5 ± 1	5.4 ± 0.8

* Mean ± SD.

† Measured diameters of 10 of the smallest cells present in each post-inversion embryo.

The most instructive subset of these heat-shocked embryos were 28 individuals that were heat shocked at the 64-cell stage and then subsequently completed an average of less than one division per cell. These 28 embryos—each of which had just completed a stereotyped set of asymmetric divisions and produced ~16 “gonidial initials” at the time it was heat shocked—went on to produce adults containing an average of 57 gonidia apiece. In all cases that could be analyzed in detail it was established that there was a direct correlation between the number of times a cell had divided after heat shock and the phenotype that it subsequently developed. Two particularly noteworthy individuals produced gonidia from all 63 cells that never divided again after heat shock, plus 12 somatic cells in one case, and 13 in the other, from the progeny of a single cell that divided 3–4 times. Another individual produced 24 gonidia from the 24 cells that did not divide again, 66 gonidia from 33 cells that divided just once after heat shock, and 28 somatic cells from the seven cells that happened to divide twice. Since somatic cell initials that cease cleaving at the seventh division cycle have an average diameter of ~8 μm , but ones that complete an eighth division have an average diameter of only ~6.5 μm (Table I), these results are consistent with Pall’s hypothesis that cells with a diameter of 8 μm or greater at the end of cleavage differentiate as gonidia.

Embryos of a Gonidialless Mutant. As a consequence of their *gls* mutation, embryos of the Gls/Reg (Gonidialless/regenerator) strain fail to divide asymmetrically, and each produces a cohort of cells of similar size that all differentiate as somatic cells (Kirk et al., 1991). Despite its lack of true gonidia, this strain can be maintained because it also carries a mutation of the *regA* (somatic regenerator) locus, which causes fully differentiated somatic cells to dedifferentiate and redifferentiate as reproductive cells that resemble normal gonidia in all regards except for their somewhat smaller size (Starr, 1970; Huskey and Griffin, 1979; Kirk et al., 1991; Tam and Kirk, 1991). This genetically gonidialless strain appeared to provide a particularly good opportunity to test the hypothesis that when cleavage is interrupted experimentally, all cells above some threshold size will develop as gonidia.

Gls/Reg cells that had completed their redifferentiation and begun to divide were sorted by cleavage stage and subjected to heat shock either in bulk (method 4) or on the microscope stage (method 1). The two methods yielded similar results. Like wild-type embryos, Gls/Reg embryos ceased dividing during the heat shock and then divided only a few times after the heat shock was terminated. Fortunately, however, analysis was simplified because Gls/Reg embryos exhibited little or no intra-individual variation in the number of cell divisions completed after termination of heat shock. Thus, when Gls/Reg embryos were heat shocked at the 8-, 16-, or 32-cell stage, typically all cells divided either one or two more times, generating juvenile spheroids containing 16, 32, 64, or 128 cells.

Of these heat-shocked specimens, all that reached the 128-cell stage differentiated like untreated Gls/Reg embryos: that is to say, all of their cells first differentiated as somatic cells (Fig. 7 A) and only a day later began to redifferentiate as gonidia (Fig. 7 B). In marked contrast, none of the cells of individuals that ceased cleaving at the 16- or 32-cell stage, and none of the cells in 44 out of the 46 individuals that ceased cleaving at the 64-cell stage, ever developed any somatic cell features such as eyespots or flagella. Instead, all cells of all 44 of these individuals developed directly into gonidia that enlarged greatly within the first day (Fig. 7 C) and cleaved within two days (Fig. 7 D). These two distinct categories of results are underscored by the results obtained with 42 Gls/Reg embryos that were heat shocked at the 32-cell stage. Of these 42 embryos, 11 completed two additional divisions after the end of the heat shock, reached the 128-cell stage, and produced only somatic cells; the remaining 31 embryos reached only the 64-cell stage and produced only gonidia. Since the cells of 64-cell Gls/Reg embryos average ~8 μm in diameter and those of 128-cell embryos average ~6 μm in diameter (Table III), these results are also consistent with the notion that the minimal cell diameter for gonidial specification in *Volvox* may lie in the range of 8 μm . Interestingly, this notion was also reinforced by the two exceptional 64-cell juveniles (of the 46 mentioned above) in which all cells differentiated as somatic cells rather than as gonidia. These exceptional juveniles were derived from cells that were <35 μm in diameter at the time cleavage was initiated (which is significantly below the mean, see Table III) and had correspondingly small cells (average cell diameter <7 μm) at the 64-cell stage.

Cell Size vs. Cell Fate After Microsurgical Modification of Wild-type Embryos

Blastomere Isolations. During normal embryogenesis of wild-type *V. carteri* f. *nagariensis*, the potential to form gonidia appears to be segregated at the fourth division: cells derived from the eight anterior blastomeres of the 16-cell embryo normally cleave asymmetrically (at the 32-cell stage) to produce two gonidia each, whereas descendants of the eight posterior blastomeres cleave symmetrically at all divisions and produce no gonidia (Starr, 1969, 1970; Green and Kirk, 1982). To determine whether this pattern is a consequence of a fundamental difference in anterior vs. posterior cytoplasm (as was shown to be the case in *V. obversus*;

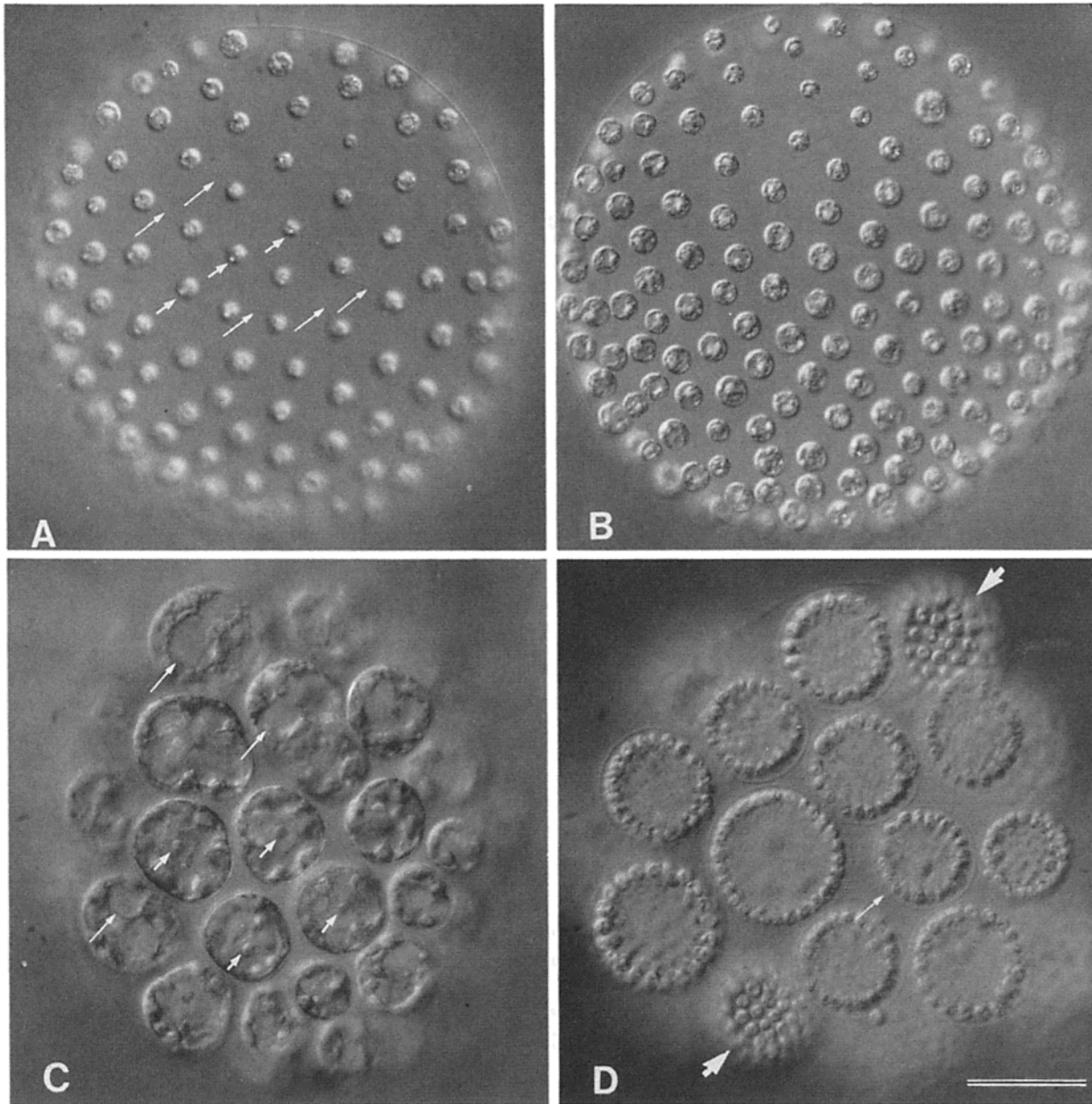


Figure 7. The effects of heat shock on cleaving Gls/Reg embryos. (A) A spheroid derived from a Gls/Reg embryo that was heat shocked one day earlier at the 64-cell stage and that cleaved two more times after the end of heat shock. All cells have developed diagnostic features of somatic cells such as flagella (*longer arrows*) and eyespots (*shorter arrows*). (B) An individual similar to that in A, but photographed two days after the end of heat shock. By now, as a consequence of their *regA* mutation, cells have begun to enlarge and redifferentiate as gonidia. (C) A spheroid derived from a Gls/Reg embryo that was heat shocked 1 d earlier at the 16-cell stage and that cleaved one more time after the end of heat shock. None of the cells have developed somatic cell features; instead they have developed the diagnostic features of gonidia, such as large peripheral vacuoles (*longer arrows*) and large central nuclei with prominent nucleoli (*shorter arrows*). Compare with A. (D) An individual similar to that in C, but photographed two days after the end of heat shock. By now all gonidia have cleaved to produce normal Gls/Reg juveniles containing flagellated somatic cells (*small arrow*), and two progeny spheroids (*large arrows*) are already in the process of hatching and swimming away. Compare with B. Nomarski differential interference contrast microscopy. Bar, 50 μm .

Ransick, 1991) we separated anterior from posterior blastomeres after the fourth division (Fig. 8 A) and followed their subsequent development by method 2 or method 4.

The subsequent cleavage behavior of isolated blastomeres differed from what would have been seen had they been left in the intact embryo. Most isolated blastomeres produced

one (or occasionally two) large cell(s) through a combination of asymmetric division and premature cessation of division (Fig. 8, B–D), and all such large cells later developed gonidial morphology (Fig. 8, E and F) and divided to produce normal progeny (not shown). The number of gonidia produced by anterior blastomeres was not significantly

Table III. Estimated Sizes of Cells at Each Stage of Cleavage In Gls/Reg Embryos

Cleavage stage	Average diameter	Volume/cell	Total volume	Percent of gonidial volume
	μm^*	$\mu\text{m}^3\dagger$	μm^3	
Uncleaved "gonidium"§	40	33,500	33,500	100
I (2-cell)	25	7,950	15,900	47
II (4-cell)	20	4,200	16,800	50
III (8-cell)	15	1,900	15,400	46
IV (16-cell)	12	1,050	16,800	50
V (32-cell)	10	500	16,100	48
VI (64-cell)	8	270	17,300	52
VII (128-cell)	6	120	15,400	46
VIII (256-cell)	5	64	16,400	49

* Regenerated cell diameter by direct measurement, other diameters calculated from estimated volumes, assuming spherical cells.

† Estimated from measurements of light micrographs of cleaving embryos.

§ From a regenerated somatic cell.

different from the number produced by posterior blastomeres: 66 anterior blastomeres monitored by method 4 produced a total of 80 gonidia (range, 0–4; mean 1.11; standard deviation, ± 0.69), whereas 65 posterior blastomeres produced 68 gonidia (range, 0–3; mean, 1.05; standard deviation, ± 0.51).

Cytoplasmic Ligation. Ransick (1991) used "posterior ligation" of uncleaved *V. obversus* gonidia as a way to generate large cells in an embryonic region that normally produces only somatic cells. He observed that such posterior large cells never developed as gonidia, and used this result to support the conclusion that in *V. obversus* both a minimum cell size and a specialized type of anterior cytoplasm were required for a cell to develop as a gonidium. To determine whether this conclusion could be extended to *V. carteri*, similar operations were performed here on *V. carteri* embryos (Fig. 9).

Because of technical difficulties and high mortality rates

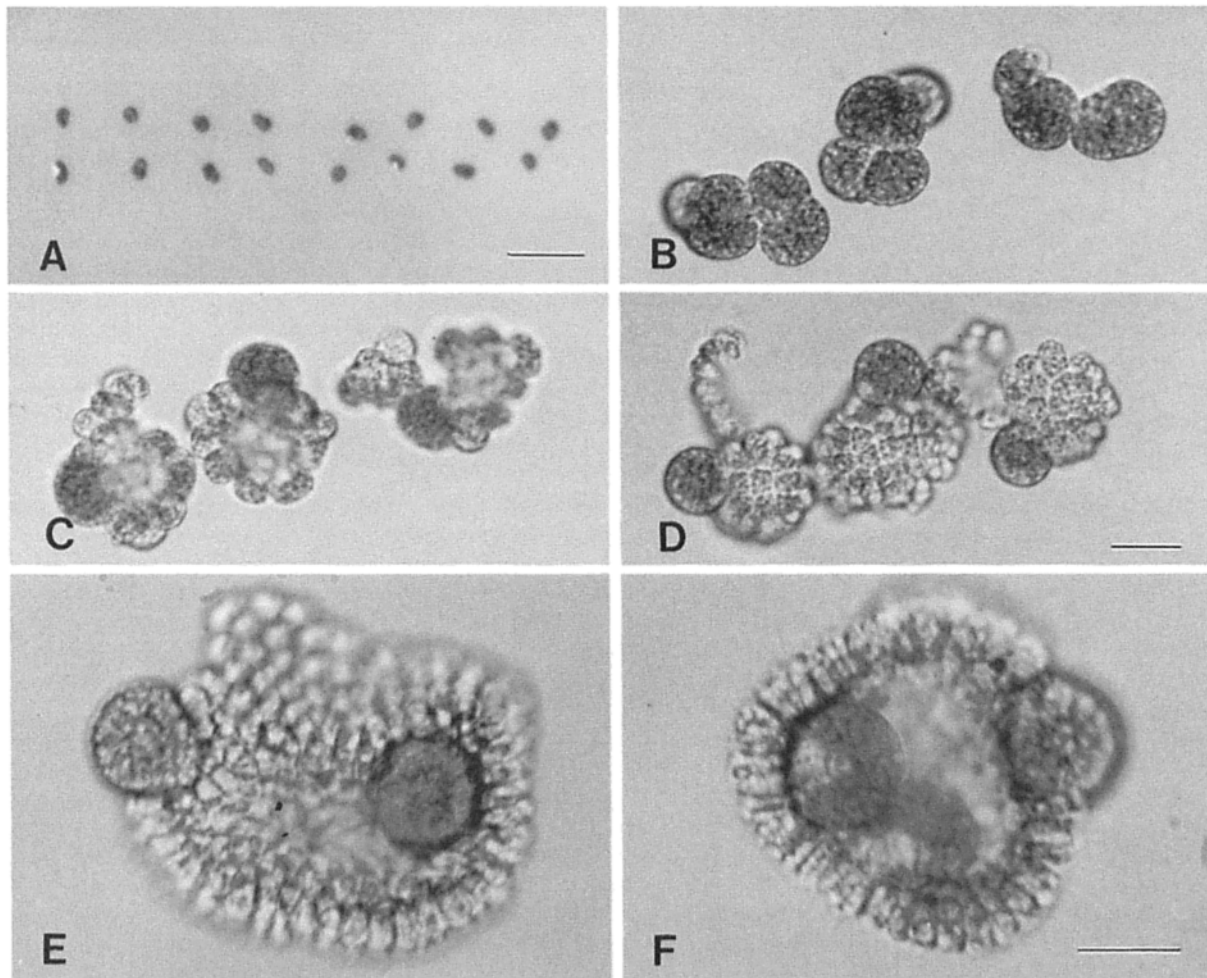


Figure 8. Development of wild-type blastomeres isolated at the 16-cell stage. (A) A set of 16 isolated blastomeres photographed through the dissection microscope immediately after the dissection. (Upper row) posterior blastomeres; (lower row) corresponding anterior blastomeres. (B–D) Three posterior blastomeres monitored by method 2 and photographed at successive cleavage stages. Note that each blastomere has produced one large cell through a combination of asymmetric division and early cessation of division. None of the cleavage patterns are entirely normal for such blastomeres. (E) A newly inverted juvenile, containing two gonidia, derived from an anterior blastomere. (F) A similar juvenile derived from a posterior blastomere. Bars: (A) $\sim 100 \mu\text{m}$; (B, C, and D) $25 \mu\text{m}$; (E and F) $10 \mu\text{m}$.

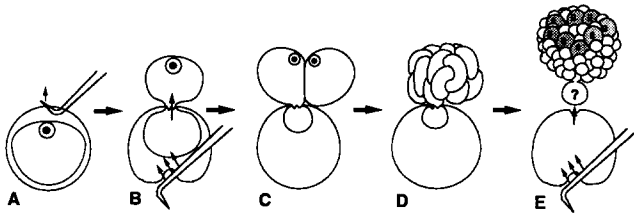


Figure 9. The design of the cytoplasmic-ligation experiments. Just prior to cleavage, gonidia contract and flatten on their anterior surface (which is defined by the location of the nucleus). At this stage a hole was made in the anteriormost region of the surrounding glycoprotein vesicle with a bent tungsten microneedle (A); pressure was then applied to the posterior region of the vesicle so as to partially expel the gonidium (B). The drawing shows the desired outcome, in which the gonidium was expelled along its anterior-posterior axis and the resulting cytoplasmic lobe was located near the posterior pole; however, in many cases the gonidium rotated during expulsion, resulting in the cytoplasmic lobe being located too far from the posterior pole to provide a useful test case. The nucleated, expelled portion of the gonidium now begins to cleave (C and D), while the cytoplasmic lobe retained within the vesicle remains undivided but attached to one blastomere by an isthmus. (E) After the asymmetric division that sets apart the true gonidial initials (g), pressure was once again applied to the vesicle to expel the cytoplasmic lobe from the vesicle; as cytoplasm from this lobe becomes incorporated into the cell to which the lobe has remained attached, a large posterior cell is generated. The developmental potential of this cell can now be assessed.

at several stages of the operation, many more than 100 ligations had to be performed to generate 10 successful cases in which a viable, enlarged cell was produced in an area that normally produces only somatic cells, and in which the organism survived through at least one full asexual life cycle. Three features facilitated monitoring the development of the large cells that were generated by this method: (a) These cells were much denser in appearance and more intensely green than any of the other cells, and therefore remained clearly identifiable throughout development (Fig. 10, D and E). (Presumably this dense, green appearance reflects the fact that the posterior cytoplasm of an uncleaved gonidium that is included in such a ligated lobe is extremely chloroplast rich; see Green et al., 1981.) (b) Often the enlarged cell divided asymmetrically in such a fashion that it created a surrounding cluster of slightly larger-than-normal somatic cells that helped to confirm the identification of the cell of interest. (c) Usually the large, dark cells created by these manipulations were carried to the interior of the juvenile during embryonic inversion; but in two fortuitous cases the large cell created by ligation detached from its host embryo during inversion but remained viable (Fig. 10 G, inset). These two detached cells were recovered and cultured in isolation, which permitted their development to be followed unambiguously (Fig. 10 G).

In all 10 successful test cases (including the two cases in which the test cells detached during inversion) the experimentally generated large cells cleaved to produce progeny, indicating clearly that they had differentiated as gonidia. In none of these cases was the cleavage pattern of the experimentally generated gonidium entirely normal; therefore, the juveniles that they produced ranged from slightly (Fig.

10 F) to highly (Fig. 10, G and H) unusual in their organization. Nevertheless, nearly all cells derived by division of the experimentally enlarged cells differentiated as either somatic cells or gonidia. Three of these individuals with highly abnormal morphology were followed for an additional generation and were observed to produce essentially normal third-generation offspring (Fig. 10 I).

Discussion

Although a number of different asymmetric division patterns characterize the asexual and sexual development of wild-type *V. carteri* (Starr, 1969, 1970) and various mutants (Kirk et al., 1991), in all cases there is a one-to-one correlation between the large cells that are created by asymmetric division and the cells that subsequently develop as germ cells. Here we used several different approaches to evaluate the causal significance of this relationship.

Cell Size Is the Only Parameter That Is Regularly Correlated with Cell Fate

Pall (1975) first suggested that "some cell parameter closely related to cell size" is critical for gonidial specification in *V. carteri* on the basis of a correlation he observed between the sizes of cells present when cleavage was terminated precociously in *pcd* mutants and the proportion of all cells that subsequently became gonidia. We attempted to test that conclusion by tracking the developmental fates of individual cells of known size in two mutants that resembled Pall's *pcd* mutants. In both of these mutants individual cells withdrew from the cleavage program prematurely in a wholly unpredictable fashion, resulting in juveniles that contained cells of an assortment of sizes in no discernible or reproducible spatial pattern (Fig. 1). Despite the absence of any temporal or spatial pattern in which cells of various sizes were produced, a simple relationship was observed: all cells that were $>9 \mu\text{m}$ in diameter at the end of cleavage developed as gonidia, all cells below $6 \mu\text{m}$ at the end of cleavage developed as somatic cells, and cells between 6 and $9 \mu\text{m}$ in diameter exhibited a strongly increasing tendency to develop as gonidia with increasing size (Fig. 2). This study not only provided qualitative support for Pall's (1975) conclusion that cell size at the end of cleavage is causally linked to cell fate, it also provided quantitative support for his conclusion that the minimum size required to specify a reproductive fate was a diameter of $\sim 8 \mu\text{m}$ at the end of cleavage.

Similar results were obtained in studies of a mutant (S16) that exhibits temperature-sensitive abnormalities of the cyto-kinetic apparatus (Mattson, 1984), such that at elevated temperatures supernumerary large cells are created in an apparently random pattern (Fig. 3). Although S16 embryos cleaving at elevated temperatures produced large cells with no discernible regard for the territories in which gonidia and somatic cells are normally found, the proportion of cells that developed as gonidia was strongly correlated with the proportion of cells that had been $>8 \mu\text{m}$ in diameter at the end of cleavage (Fig. 4). (With increasing temperature, the number of cells above $8 \mu\text{m}$ in diameter at the end of cleavage increased slightly more rapidly than the number of gonidia. This is due to the fact that with increasing cleavage temperature increasing numbers of cells of indeterminate phenotype

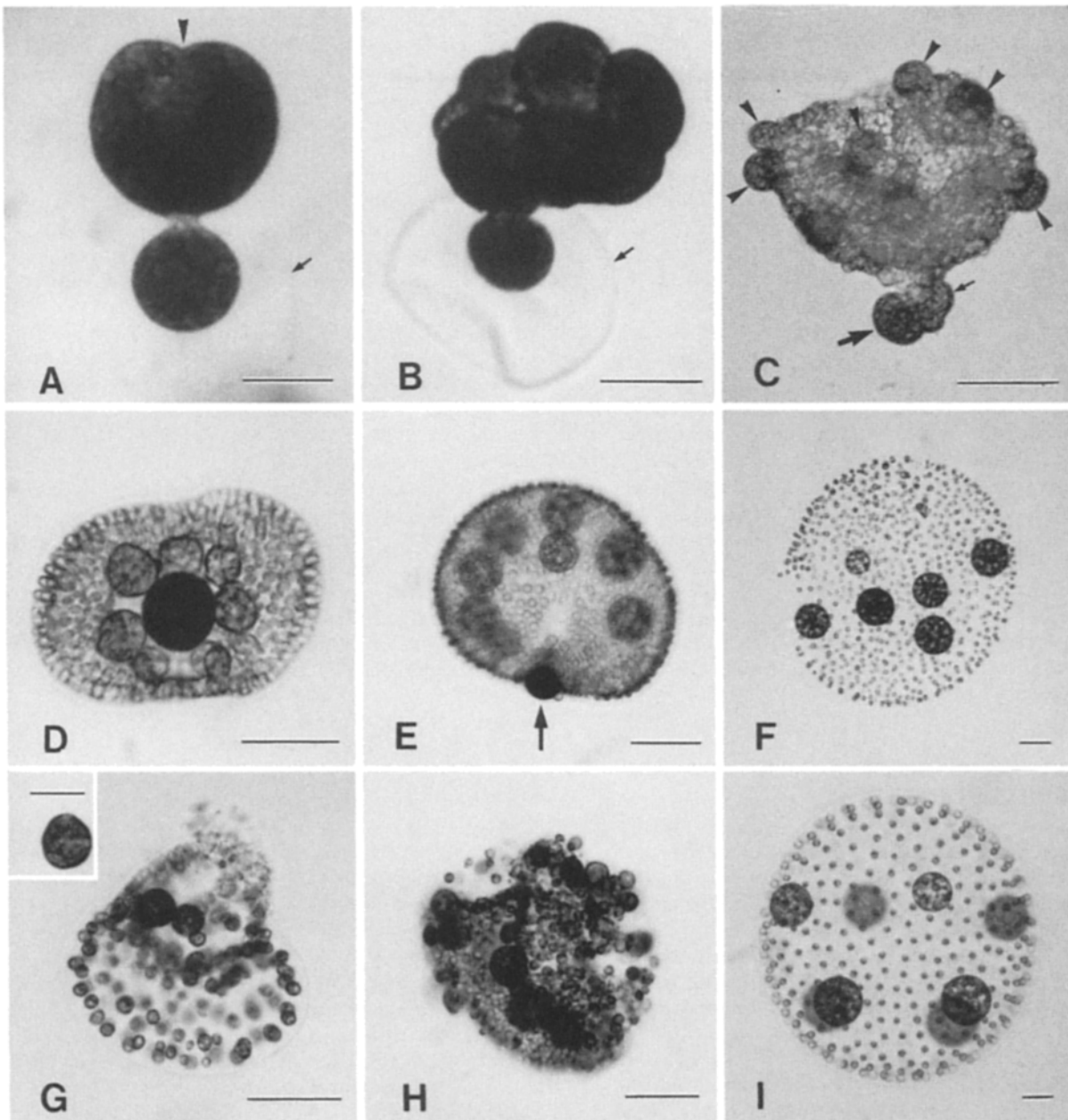


Figure 10. Representative embryos and progeny produced in posterior ligation experiments. (A) A gonidium just after formation of a cytoplasmic lobe. The first cleavage furrow that is forming (*arrowhead*) identifies the anterior pole of the embryo and indicates that the cytoplasmic lobe is located near the posterior pole. The glycoprotein vesicle that acts as a noose to retain the cytoplasmic lobe (see Fig. 9 B) is just barely visible (*arrow*). (B) A similar embryo, following three cleavage divisions. The vesicle (*arrow*) is clearly seen. The cup-shaped opening characteristic of the anterior end of an 8-cell embryo can be seen near the top, and indicates that the cytoplasmic lobe is located near the posterior end of the lower left blastomere. (C) An embryo that has continued to cleave after its posterior cytoplasmic lobe was released from the vesicle at the 64-cell stage. Several “true” gonidia can be seen around the anterior hemisphere (*arrowheads*). The cell that absorbed the posterior cytoplasmic lobe appears to have undergone asymmetric division to form a number of smaller cells (*small arrow*) and one large, dense cell (*larger arrow*). (D) An optical section through a juvenile very shortly after the completion of inversion. In this case the large cell created by cytoplasmic ligation was located in the anterior hemisphere and it has ended up in the middle of a cluster of gonidia. Although this cell was produced too far from the posterior pole to provide a useful test case, it remained identifiable throughout its development and subsequent cleavage because of its greater density. (E) A juvenile that has inverted and begun to expand, and that contains one large, dense posterior cell created by cytoplasmic ligation (*arrow*). This cell subsequently cleaved to produce the juvenile shown in H. (F-H) Representative progeny, with varying degrees of organizational abnormalities, that were derived from experimentally generated large posterior cells. The juvenile in G was derived from an experimentally generated large posterior cell (shown in the inset) that detached from the rest of the embryo during inversion, was recovered, and was cultured in isolation. (I) One of seven relatively normal third-generation spheroids produced by the misshaped second-generation individual shown in H. Bars: (A-I) 25 μm ; (inset in G) 10 μm .

were produced. This phenomenon, which is rarely observed in other strains of *V. carteri*, is probably related to the fact that S16 has been observed to produce both anucleate and multinucleate cells at these elevated temperatures [Mattson, 1984].)

While the findings of the preceding two analyses are consistent with Pall's hypothesis that cell size at the end of cleavage is a cause of cell specification, both are also consistent with the opposite hypothesis: that it is an effect. That is to say, these studies are also consistent with the hypothesis that when a cell is specified as a potential gonidium it activates a division program that leaves it $>8 \mu\text{m}$ in diameter at the end of cleavage. For example, it might be imagined that one function of the putative germ plasm of *Volvox* (Yates and Kochert, 1970) might be to activate a division program involving asymmetric divisions and premature cessation of division that would leave cells containing germ plasm larger than sister cells lacking it. By such a model, it might then be postulated that the cleavage abnormalities in the Pcd-K1, S1s-S1, and S16 strains are a consequence of abnormalities in the distribution of germ plasm. While a number of considerations seemed to us to make such an interpretation unlikely (e.g., earlier observations that the S16 mutation affects basic morphological features of the cytokinetic apparatus; Mattson, 1984), we felt that it was necessary to test Pall's hypothesis more directly by producing large cells that contained only cytoplasm of the type normally associated with a somatic cell fate. The remainder of the experiments reported here were designed to meet that objective.

Heat shock provided a way to truncate the cleavage program without blocking subsequent development. Analysis of 158 embryos that were heat shocked at the 32- or 64-cell stage revealed a strong, inverse correlation between the number of cells present at the end of cleavage and the number of gonidia produced: the fewer cells produced (and hence the greater their average size), the greater the number that developed as gonidia. Although these aggregated data indicated that there is a relationship between cell size and cell fate in *V. carteri*, the details of the relationship become much clearer when they are disaggregated.

The subset of heat-shocked embryos that speak most directly to the question of what role asymmetric division plays in gonidial specification is the group of 28 that were heat shocked at the 64-cell stage and that subsequently divided less than once per cell, on average. If the function of asymmetric division were to segregate to the larger cells a "germ plasm" that is essential for gonidial specification, then 64-cell embryos (which had just executed a round of asymmetric division to produce ~ 16 large "gonidial initials") should not be capable of producing more than ~ 16 gonidia each. In fact, however, these embryos generated an average of 57 gonidia each. The two individuals in which 63 cells that failed to divide after the termination of heat shock all developed as gonidia indicate clearly that the potential to form gonidia is not segregated at the asymmetric sixth division, but is retained by all cells at the 64-cell stage. By corollary, the exceptional cell within each of these embryos that divided 3–4 times to produce 12 or 13 somatic progeny indicates with equal clarity that whether any cell will express the gonidial potential that it still possesses at the 64-cell stage—or will lose that potential and produce only somatic progeny—depends on the details of its subsequent cleavage be-

havior. This interpretation was reinforced and extended by the individual in which all 66 cells derived from the 33 blastomeres that divided once after the end of heat shock (as well as all 24 cells that did not divide at all) developed as gonidia, whereas all seven cells that divided twice produced only somatic progeny.

The conclusions derived from these studies of wild-type embryos were further reinforced by the results obtained when cleavage of Gls/Reg embryos was interrupted by heat shock. When cleavage of Gls/Reg embryos is allowed to run its course, all cells differentiate all of the visible features of somatic cells. (The reason that these cells later redifferentiate to function like gonidia is that the *gonidialless* mutation can only be maintained in combination with a *regA* mutation, which by itself confers on somatic cells the ability to "regenerate" [i.e., redifferentiate as gonidia; Starr, 1970; Huskey and Griffin, 1979; Kirk et al., 1991; Tam and Kirk, 1991].) Although embryos of this mutant normally produce no cells that differentiate as "true" gonidia, in nearly every case they produce nothing but cells that develop immediately as gonidia if cleavage is interrupted at or before the 64-cell stage by heat shock (Fig. 7, C and D). In contrast, in every case in which cleavage proceeded to or beyond the 128-cell stage, all Gls/Reg cells developed unambiguous somatic cell features (Fig. 7 A) and only redifferentiated as gonidia (as a consequence of their *regA* mutation) more than a day later (Fig. 7 B).

In short, these studies demonstrated that a strain that has previously appeared to be genetically incapable of forming true gonidia produces nothing but gonidia when cleavage is interrupted early enough to leave all cells $>\sim 8 \mu\text{m}$ in diameter (Table II). When viewed strictly from the point of view of trying to distinguish between the "cell size" (Pall, 1975) and the "germ plasm" (Kochert, 1975) models for gonidial specification, this result seems to argue rather forcefully for a causal role of cell size in the gonidial specification process.

There are, however, certain alternative interpretations of the heat shock results that should be considered. (a) Might it be that—rather than acting on the gonidial specification process through its effect on cell division and cell size—heat shock acts directly on the gene expression system, causing the gonidial program to be expressed in cells that would otherwise not have expressed it? The fact that not all cells that were subjected to the same thermal stress at the same time experienced the same change in gene expression might, perhaps, simply be attributed to some sort of stochastic behavior. However, such an explanation would not seem to account for the fact that the way in which each cell responded was so strongly correlated with its post-heat shock cleavage behavior and its resultant size (Fig. 6; Table II). The strength of this correlation is demonstrated most clearly by the results obtained when Gls/Reg embryos were heat shocked at the 32-cell stage: all 31 such embryos that subsequently divided only one more time produced only gonidia, whereas 11 equivalent embryos that happened to divide a second time after the end of the heat shock produced only somatic cells. (b) In light of the apparent strength of the relationship just cited, might it be that it is the number of divisions that a cell has completed—rather than its size at the end of division—that determines which developmental program is activated? A major weakness of such a "division-counting" hypothesis

is that it does not provide any simple or obvious way to unify the following observed facts: in normal *Volvox* development a gonidial initial completes nine divisions, but in the heat shocked 64-cell wild-type embryos studied here, many (if not all) somatic initials that completed only eight divisions developed as somatic cells. Moreover, in every heat-shocked Gls/Reg embryo that was examined in this study, every cell that completed seven or more divisions developed as a somatic cell. And in two heat-shocked Gls/Reg embryos that were studied here (the two exceptional 64-cell individuals that were derived from smaller-than-normal cleaving cells) all cells differentiated as somatic cells after completing only six divisions. Indeed, the only parameter we can find that ties all of these observations together is the size of the cells at the end of division. Each of the cases cited is consistent with Pall's hypothesis that the minimum diameter required for specification as a gonidium is $\sim 8 \mu\text{m}$. The various cases differ from one another, however, in the number of divisions that are required to pass below that threshold.

Despite the apparent strength of the size/fate relationship in all the studies discussed to this point, we felt that to test this relationship more directly it would be desirable to use alternative methods of producing large cells in "normally somatic" embryonic regions that did not involve either mutations or experimental treatments whose primary cytological effects were indeterminate. To this end, two kinds of microsurgical approaches were used.

In normal development of *V. carteri* the potential to divide asymmetrically and produce gonidia appears to be segregated asymmetrically to the anterior eight blastomeres formed at the fourth division cycle. However, when anterior and posterior blastomeres of wild-type embryos were separated from one another at the 16-cell stage, they exhibited similar patterns of asymmetric division to one another and eventually produced similar numbers of gonidia (1.11 ± 0.69 vs 1.05 ± 0.51 , respectively). The observation that both anterior and posterior blastomeres executed different patterns of cleavage in isolation than they would normally execute in the intact embryo suggests that the stereotyped temporal and spatial pattern of asymmetric divisions that is normally observed in an intact *V. carteri* embryo may be regulated by cell-cell interactions of some type (whether structural or chemical) within the embryo as a whole. Be that as it may, in the present context the most important conclusions to be drawn from these blastomere isolation experiments are that (a) neither the ability to divide asymmetrically, nor the ability to produce gonidia is a distinctive property of anterior blastomeres at the 16-cell stage, and (b) when large cells are produced by blastomeres containing cytoplasm that would normally be used to produce only somatic cells, those large cells become gonidia.

These conclusions were reinforced by the results of all 10 cases in which cytoplasmic ligation (Fig. 9) was successfully used to produce large posterior cells. Despite the fact that each of these experimentally generated large cells contained only posterior cytoplasm that is normally associated with cells of somatic fate, they uniformly failed to develop any of the diagnostic features of somatic cells. Instead, each eventually divided and produced an embryo. This result clearly indicates that any *V. carteri* cell that possesses sufficient cytoplasm, even if it is of a posterior type, can differentiate as a gonidium.

It is of passing interest that these experimentally generated large cells divided abnormally to produce juveniles of unusual cellular organization (Fig. 10, F-H). This outcome suggests that large cells that possess mostly posterior cytoplasm may activate the gonidial program of gene expression that leads to the initiation of embryogenesis, even though they lack the cytoplasmic organization necessary for a normal pattern of cleavage divisions. We believe, however, that the atypical cleavage behavior exhibited by these large posterior cells should not be permitted to diminish the significance of the central fact that such cells do cleave, which is the hallmark of gonidial cells.

In summary, the experiments reported here provide no support for the hypothesis that gonidial specification in *V. carteri* f. *nagariensis* requires any specialized kind of cytoplasm that is of restricted distribution in the embryo. Nor are they consistent with the hypothesis that embryonic cells of *V. carteri* possess a division-counting mechanism, and that it is simply the number of divisions each cell has completed that determines which of the two alternative developmental programs it will activate. Rather, all of the present results are consistent with the hypothesis of Pall (1975) that cells of an asexual embryo of *V. carteri* f. *nagariensis* that are above a threshold size at the end of embryogenesis—wherever and however they have been produced—develop as gonidia, whereas cells of lesser size develop as somatic cells. Moreover, these studies all support the conclusion that the threshold size required at the end of cleavage for activating the gonidial program of development is, as Pall (1975) estimated, a diameter of $\sim 8 \mu\text{m}$. Interestingly, this is also the minimum size that Ransick (1991) found was essential for activating the gonidial program of development in *V. obversus*.

Cell Size as a Regulator of Gene Expression: a Working Hypothesis

The fact that the present study has found no evidence for a regionally specialized cytoplasm with a unique role to play in gonidial determination does not imply that there are no cytoplasmic factors in the cleaving embryo that are crucially important for activating the gonidial program of development. It is highly probable that there are such factors. But the present studies suggest that such factors must be rather evenly distributed throughout the embryo, and only exert their determinative influence in cells above a threshold size.

Many classical and contemporary studies indicate clearly that various eukaryotic cells have mechanisms for assessing their nucleo-cytoplasmic ratio, or their volume, or some other attribute of cell size, and modulating their behavior accordingly (for examples see Fankhauser, 1945; Prescott, 1956; Johnston et al., 1977; Newport and Kirschner, 1982; Edgar et al., 1986; Hayles and Nurse, 1986; Cossins, 1991; Henery, et al., 1992). However, in contemplating how factors required for dichotomous differentiation of two cell types might be uniformly distributed in a predivision cell, but then be activated differentially in daughter cells of different size, we are particularly struck by a possible precedent provided by sporulating *Bacillus subtilis* cells, in which asymmetric septation appears to trigger two different patterns of gene expression and development in the two daughter cells (Kunkel, 1991; Stragier, 1991).

After asymmetric septation of starved *B. subtilis* cells, a

gene expression cascade is activated that eventually results in the smaller cell becoming a spore (a "germ cell" with reproductive potential) while the larger cell develops as a spore mother cell (a terminally differentiated "somatic" cell). A key factor required to initiate this gene expression cascade is σ^F , a protein specifically required for the transcription of spore-specific genes. One current model to explain how σ^F is activated (Margolis et al., 1991) is as follows: before septation σ^F is synthesized in an inactive form and is uniformly distributed in the cell, as are two activators and one inhibitor of σ^F ; the inhibitor of σ^F is dominant, and thus σ^F remains inactive. Upon asymmetric septation, however, the membrane/cytoplasm ratio of the small cell rises sharply, increasing the abundance of one of the activators (a membrane component) relative to the inhibitor (a cytoplasmic component). It is postulated that it is this increase in the activator/inhibitor ratio in the small cell that leads to activation of σ^F and initiation of spore-specific gene expression (Margolis et al., 1991). Subsequently, one of the spore-specific gene products acts across the septation membrane to activate—selectively within the larger cell—a second σ factor that was also uniformly distributed in an inactive form before division (Kunkel, 1991). This second σ factor, which is required for transcription of spore mother cell-specific genes, then activates the rest of the genetic program that leads to development of two entirely different phenotypes by sister cells that initially differed only in size.

Any number of similar models could be constructed for *V. carteri* in which differences between the membrane/cytoplasm ratios, or the cytoplasm/nuclear ratios, of the small and large cells produced by asymmetric division would be postulated to trigger a similar cascade of differential gene expression. The challenge will be to find ways of testing such models. However, whatever the initial steps might be by which a difference in cell size is transduced into a difference in gene expression in large and small *V. carteri* cells, regulatory genes that are reasonable candidates for such differential expression have already been identified and incorporated into a working hypothesis regarding the genetic control of germ/soma specification in *V. carteri* (Kirk, 1988; Tam and Kirk, 1991). With the present results incorporated, this hypothesis is as follows: (a) The "default" pathway of development in *V. carteri* is one in which all cells initially express both "somatic" and "gonidial" genes simultaneously, and as a result differentiate first as somatic cells and then redifferentiate as gonidia. (This is the visible pattern of development observed in many primitive relatives of *Volvox*, and in certain double mutants of *V. carteri*; Tam and Kirk, 1991). (b) In *V. carteri* cells $> \sim 8 \mu\text{m}$ in diameter a set of loci known as the *lag* ("late gonidia") genes (Kirk, 1990) are expressed and the *regA* gene is repressed, whereas in cells below this size the opposite is true: the *lag* genes are repressed and *regA* is expressed. (c) The *lag* genes act in the large cells to prevent expression of somatic genes, whereas the *regA* gene acts in the small cells to prevent expression of gonidial genes. Molecular evidence consistent with this hypothesis has been obtained (Tam and Kirk, 1991). A corollary to this hypothesis is that it is because they lack the *regA* function (and thus cannot repress gonidial gene expression) that the somatic cells of *regA*⁻ mutants are able to redifferentiate as gonidia, and thus provide an exception to the rule that a cell must be above a certain size to express the gonidial program of develop-

ment. Similarly, this hypothesis accounts for the fact that in *lag*⁻ mutants the large cells produced by asymmetric division do not differentiate directly as gonidia: because they lack the *lag* functions required to repress somatic genes, they first differentiate as larger-than-normal somatic cells before redifferentiating as gonidia (Kirk, 1990; Kirk et al., 1992).

The Evolution of Cell Size as a Determinant of Cell Fate

It is particularly interesting and worthy of separate comment that the microsurgical manipulations that were used to analyze gonidial specification in *V. obversus* (Ransick, 1991, 1993) led to different results and conclusions when applied in the same manner to *V. carteri* embryos in the present study. With *V. obversus*, both blastomere isolation and posterior ligation experiments supported the conclusion that gonidial potential in *V. obversus* is a unique property of cells containing cytoplasm from the anteriormost region of the embryo (Ransick, 1991, 1993). When applied here to *V. carteri* f. *nagariensis*, in contrast, the same two operations led to the conclusion that all regions of the embryo have equivalent potential to form gonidia, and that the size of cells at the end of cleavage appears to be the principal determinant of cell fate. This raises questions about the ways in which different mechanisms of germ-cell specification may have arisen during *Volvox* evolution.

In this context it is important to note that no asymmetric divisions occur in *Pleodorina* (the genus usually regarded as the closest relative of the genus *Volvox*) or in most species of *Volvox*, including a number of species that are thought to be closely related to *V. obversus* and *V. carteri*, such as *V. powersii*. In all of these forms, gonidia and terminally differentiated somatic cells do eventually become differentiated from one another in relatively predictable patterns, but all gonidia are developed from cells that were initially indistinguishable from somatic cells, but then undergo enlargement and differentiation. In such forms gonidial specification obviously is not mediated by initial differences in cell size, but must have some other basis, possibly a difference in cytoplasmic quality between cells that appear identical. Given its widespread occurrence in the genus *Volvox* and its relatives, such a mechanism of gonidial specification appears to be primitive, whereas the mechanisms involving asymmetric division that are used by *V. obversus* and *V. carteri* appear to be derived (compare Desnitski, 1992). Comparative analyses of ribosomal RNA sequences have indicated that *V. carteri* and *V. obversus* are relatively closely related to one another and to *Pleodorina* (Larson et al., 1992). However, more extensive phylogenetic studies will be required to distinguish whether the differing methods of gonidial specification that *V. obversus* and *V. carteri* now exhibit may represent two successive evolutionary developments within a single lineage, or are two independently derived, and slightly different mechanisms for accomplishing similar ends.

In either case, it is of some interest to ponder what might have been the selective advantage that led to the evolution of the *V. carteri* form of gonidial specification. *Volvox* species with markedly asymmetric cleavage divisions (and hence with gonidial initials that are much larger than somatic initials) may have shorter asexual life cycles than species in

which gonidia are initially indistinguishable from somatic cells, and which therefore require a more prolonged period of development. Wild-type *V. carteri* f. *nagariensis* has the shortest asexual life cycle yet reported for the genus (2 d under optimum conditions); but in "late gonidia" mutants of this species, in which gonidial initials first differentiate as somatic cells and then redifferentiate as gonidia, the life cycle is lengthened by 50% (to 3 d) and more nearly resembles that of other *Volvox* species. Thus, one possibility is that in certain environments a gonidial developmental program involving asymmetric division followed by direct development of the large cells as gonidia was favored by natural selection because it leads to a more rapid completion of the life cycle, and thereby confers a reproductive advantage.

A very similar hypothesis regarding the selective advantage of asymmetric division has recently been proposed with respect to the evolution of molluscs and other invertebrates with spiral cleavage (Freeman and Lundelius, 1992). An important early step in the development of all spiralian embryos is the specification of the dorsal-posterior, or D, quadrant. But D quadrant specification can occur in either of two rather different ways, one of which involves asymmetry of the first two cleavage divisions and one of which involves symmetric early divisions followed by an inductive interaction at a later stage. In the view of Freeman and Lundelius (1992) the inductive method of specification is evolutionarily primitive, and the mechanism involving asymmetric division is derived. Furthermore, they postulate that the selective advantage of asymmetric division as a specification mechanism is that in all forms in which the D quadrant is initially larger than the other three quadrants of the embryo, subsequent developmental processes are accelerated, and the organism is able to condense its life history by either producing adult structures in the larva, or by bypassing the larval stage altogether. It will be interesting to learn how many developmental processes involving asymmetric division in other kinds of embryos may be amenable to a similar interpretation.

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