Evolvability, Modularity, and Individuality During the Transition to Multicellularity in Volvocalean Green Algae

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I. Overview

Evolvability, viewed as the capacity of a lineage to generate heritable, selectable phenotypic variation (Altenberg 1995; Kirschner and Gerhart 1998), is a general feature of biological life. Evolvability is thought to depend critically on the way genetic variation maps onto phenotypic variation (the representation problem) such that improvement becomes possible through mutation and selection (Wagner and Altenberg 1996). It is not known how the genotype-phenotype maps are formed nor how they are able to change in evolution and what the selective forces are (Wagner and Altenberg 1996). Properties that reduce constraints on change and allow the accumulation of non-lethal variation are thought to confer evolvability to a system (Kirschner and Gerhart 1998). One example of a such variational property is modularity (Wagner and Altenberg 1996). When defined as a genotype-phenotype map in which there are few pleiotropic effects among characters serving different functions (with pleiotropic effects falling mainly among characters that are part of a single functional complex), modularity is expected to improve evolvability by limiting the interference between the adaptations of different functions (Wagner and Altenberg 1996). Modules can be relatively easy to dissociate, recombine or redeploy in new contexts; some modules are, nevertheless, resistant to dissociation and can lead to co-variation and developmental constraints. Modular evolution may integrate previously separate functions, or create new separate modules from a formerly integrated one. How modules interact and evolve during transitions in units of evolution, or whether these interactions affect the evolutionary potential of a lineage is not yet understood.

The current hierarchical organization of life reflects a series of transitions in the units of evolution, such as from genes to chromosomes, from prokaryotic to eukaryotic cells, from unicellular to multicellular individuals, and from multicellular organisms to societies. During these evolutionary transitions, new levels of biological organization are created (Buss 1987; Maynard Smith and Szathmary 1995); moreover, individuality and new levels of heritable fitness variation have to emerge at the higher level (Michod 1999). We argue here that the emergence of individuality during the unicellular-multicellular transition requires the re-organization at the higher level of

certain basic life properties (such as immortality, totipotency, growth and reproduction). We think that the way in which this is achieved is not only instrumental for the emergence of individuality at the higher level but can also affect the potential for evolution, i.e., evolvability, of the newly emerged higher-level unit.

We suggest that during evolutionary transitions in individuality, a new genotype-phenotype map must be created, to reflect the emergence of the new higher-level unit. Furthermore, the way in which the lower-level genotype-phenotype maps are re-organized at the higher level can influence the potential for evolution of the newly emerged multi-level system. To this end, we use the volvocalean green algal group to argue that: (i) during transitions in individuality some processes have to be dissociated at the lower level and re-combined or redeployed at the higher level; (ii) the way in which certain complex sets of traits (and the genotype-phenotype maps associated with them) are re-organized during the transition affects the flexibility and robustness of the new genotype-phenotype map at the higher level, and can interfere with the potential for further evolution of the lineage; and (iii) although modularity is generally expected to improve evolvability, during transitions in individuality this expectation is complicated and sometimes compromised by constraints at the lower level.

II. The volvocalean green algal group: a case study in the transition to multicellularity

> "Few groups of organisms hold such a fascination for evolutionary biologists as the Volvocales. It is almost as if these algae were designed to exemplify the process of evolution" (Bell 1985).

1. Rationale

Our reasons for choosing the volvocalean green algal group to investigate the transition to multicellularity and individuality are three-fold. First, volvocalean green algae comprise both unicellular (*Chlamydomonas*-like) algae as well as colonial forms in different stages of organizational and developmental complexity. The so-called "volvocine lineage" contains the genus *Chlamydomonas* as well as a subset of colonial volvocalean genera that show a progressive increase

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in cell number, volume of extracellular matrix per cell, division of labor between somatic and reproductive (gonidia) cells (i.e., germ/soma separation), and proportion of vegetative cells (Larson et al. 1992) (Fig. 1). Second, multicellularity and individuality evolved multiple times in this group; the different levels of organizational and developmental complexity are thought to "represent alternative stable states, among which evolutionary transitions have occurred several times during the phylogenetic history of the group" (Larson et al. 1992), rather than a monophyletic progression in organizational and developmental complexity. Third, despite the multiple and independent acquisitions of the multicellular state and germ/soma separation in this group, none of these multicellular lineages attained high levels of complexity and/or phenotypic variability (as did other green algal lineages, especially the ancestors of land plants, the charophytes). We believe that understanding the reasons for this apparent limited spurt of diversification and complexity in this lineage will provide insight into how transitions in individuality can affect the evolvability of a lineage.
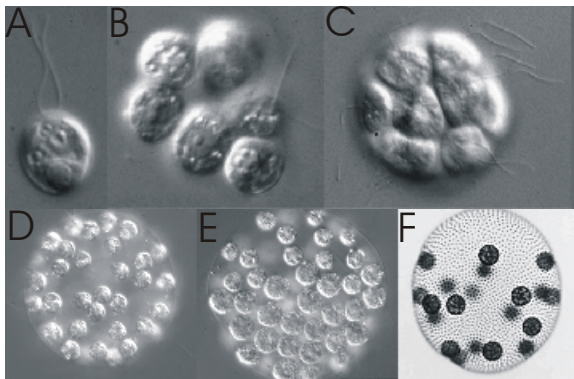

Figure 1. The "volvocine lineage": a subset of colonial volvocalean green algae that show a progressive increase in cell number, volume of extracellular matrix per cell, division of labor between somatic and reproductive cells, and proportion of vegetative cells. A: *Chlamydomonas reinhardtii*; B: *Gonium pectorale*; C: *Pandorina morum*; D: *Eudorina elegans*; E: *Pleodorina californica*; F: *Volvox carteri*. Where two cell types are present, the smaller cells are the vegetative/somatic cells, whereas the larger cells are the reproductive cells (gonidia). Images kindly provided by David L. Kirk.

2. Complexity

Many traits are known to be rather diverse in this green algal group. The observed morphological and developmental diversity among volvocalean algae appears to result from the interaction of conflicting structural/functional constraints and strong selective pressures.

All volvocalean algae share the so-called "flagellation constraint" (Koufopanou 1994), which has a different structural basis than the one invoked in the origin of metazoans (Margulis 1981; Buss 1987). In most green flagellates, during cell division the flagellar basal bodies remain attached to the plasma membrane and flagella, and behave like centrioles (which is not possible in other protists); however, in volvocalean algae, due to a coherent rigid cell wall the position of flagella is fixed and thus, the basal bodies cannot move laterally and take the position expected for centrioles during cell division while remaining attached to the flagella (as they do in other green flagellates). Therefore, cell division and motility can take place simultaneously only for as long as flagella can beat without having the basal bodies attached (i.e., only up to five cell divisions).

The presence of a coherent cell wall is coupled with the second conserved feature among volvocalean algae, which is their unique way of cell division. The volvocalean cells do not double in size and then undergo binary fission. Rather, each cell grows about 2^n-fold in volume, and then a rapid, synchronous series of n divisions (under the mother cell wall) is initiated; this type of cell division is referred as to multiple fission and palintomy (i.e., the process during which a giant parental cell undergoes a rapid sequence of repeated divisions, without intervening growth, to produce numerous small cells). Because clusters, rather than individual cells, are produced in this way, this type of division is suggested to have been an important precondition facilitating the formation of volvocacean colonies (Kirk 1998). In *Chlamydomonas*, the cells (2^2-2^4 cells) separate from each other after division. However, in many species, the cluster of 2^n cells does not disintegrate, and coenobial forms (i.e., a type of multicellular organization in which "the number of cells is determined by the number of cleavage divisions that went into its initial formation, and in which cell number is not augmented by accretionary cell divisions"; Kirk 1998) are produced. In

Gonium, the resulting cells (2^2-2^5) stay together and form a convex discoidal colony. In *Eudorina* and *Pleodorina* the cells (2^4-2^6, 2^6-2^7, respectively) are separated by a considerable amount of extracellular matrix and form spherical colonies. Finally, in *Volvox*, a high number of cells (2^{15}-2^{16}) form colonies up to 3 mm in size (Figure 1).

The two selective pressures that are thought to have contributed to the increase in complexity in all volvocalean lineages are the advantages of a large size (potentially to escape predators, achieve faster motility, homeostasis, or better exploit eutrophic conditions) and the need for motility (e.g., to access to the euphotic/photosynthetic zone) (Bell 1985). Interestingly, given the background offered by the volvocalean type of organization presented above, namely the flagellar constraint and the multiple fission type of cell division, it is difficult to achieve the two selective advantages simultaneously. As the colonies increase in size and number of cells, also does the number of cell divisions (up to 15-16 in some *Volvox* species); consequently, the motility of the colony during the reproductive phase is negatively impacted for longer periods of time than are acceptable in terms of the need to access the euphotic zone. This negative impact of the flagellation constraint is overcome by cellular specialization/division of labor: some cells are involved mostly in motility, while the rest of the cells become specialized for reproduction. The proportion of cells that remain motile throughout most or all of the life cycle is directly correlated with the number of cells in a colony: from none in *Chlamydomonas* and *Gonium*, to up to one-half in *Pleodorina* and > 99% in *Volvox* (Larson et al. 1992). In *Volvox*, the division of labor is complete: the motile (somatic) cells are sterile, terminally differentiated, and are thought to be genetically programmed to undergo cellular senescence and death once the progeny was released from the parental colony (Pommerville and Kochert 1981); only the reproductive cells (the gonidia) undergo cleavage to form new colonies (Pommerville and Kochert 1982). The present diversity in morphological and developmental complexity in the volvocalean algae reflects distinct strategies and solutions to the same set of constraints and pressures.

III. Transition in individuality during the transition to multicellularity in volvocalean green algae

In certain circumstances, a large size can be advantageous. However, cells cannot exceed a particular size because, as they increase in size, the surface/volume ratio and thus the efficiency of metabolic processes decreases. Consequently, to increase in size, the number rather than the size of cells has to increase. Groups of cells can evolve in this way. Nevertheless, the stability of such groups is low because cells can leave the group and live as free unicellular individuals. As a consequence, individuality at the higher level is difficult to achieve. However, individuality at the higher level evolved in many multicellular lineages. There are several different ways individuality can be defined, based on genetic homogeneity and uniqueness, physiological autonomy and unity, or units of selection (Michod 1999; Santelices 1999). Below, we use the physiological autonomy and unity criterion, and define an individual as the smallest unit that is physiologically and reproductively autonomous. The question we are concerned with here is: How can individuality emerge during the unicellular-multicellular transition? What are the constraints that have to be broken in order for a group to become a multicellular individual?

One can approach this question from many perspectives. Below, we present a comparative approach and focus on several general life-properties (such as growth and reproduction) and basic life-traits (immortality and totipotency). We suggest that for individuality to be created at a higher level certain processes, traits, and functions, have to be dissociated at the lower level and re-organized in new ways at the higher level. Moreover, we think that some of the differences among lineages can be explained by the way in which the re-organization of these processes and traits has been achieved during the transition to multicellularity and the emergence of individuality at the higher level. Volvocalean algae exemplify well this suggestion. In this group, the transition to multicellularity embraced unique paths, partly due to the constraints inherited from their unicellular ancestors, mainly the multiple fission type of division. Furthermore, although individuality at the higher level has been achieved in many volvocalean lineages, the way in which this was achieved interfered with the potential for further evolution of these lineages (discussed in the last section).

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To facilitate the understanding of these issues, we first discuss the concepts used in further discussion. Our goal is to pinpoint the differences in the way that various traits are expressed between unicellular and multicellular forms, and to suggest (in the next section) how they have been re-organized during the transition from unicellular to multicellular individuals. We also apply these concepts to our study case, the volvocalean green algae.

1. Unicellularity vs. multicellularity: Basic concepts

(i) General life-properties and traits

Vegetative and reproductive functions

Any biological entity features two main sets of functions, vegetative and reproductive; these basic biological functions are coupled at the level of the individual, as a functional/physiological unit. However, the two sets of functions are realized differently between a unicellular and a multicellular individual (Fig. 2A). In unicellular forms, the same cell is responsible for both vegetative and reproductive activities (i.e., they are coupled at the cell level). Nevertheless, at the level of the individual, these functions do not take place simultaneously (i.e., they are dissociated in time). In multicellular individuals with germ/soma separation, the two sets of functions are un-coupled at the cell level; some cells do only vegetative functions, whereas other cells are specialized for reproductive functions. Consequently, the two sets of functions can take place simultaneously (i.e., they need not to be separated in time anymore).

Growth is an important property of life. Interestingly, growth has different implications in unicellular vs. multicellular individuals (Fig. 2B). In the former, growth is coupled with reproduction; growth to a specific size (cell surface/volume ratio) will generally trigger the reproduction of the individual, and vice versa, reproduction requires achieving a pre-set size. In multicellular individuals, on the other hand, growth and reproduction of the individual are un-coupled; reproduction is not necessarily dependent on growth, and growth does not necessarily trigger reproduction.

(A) Unicellular — Multicellular
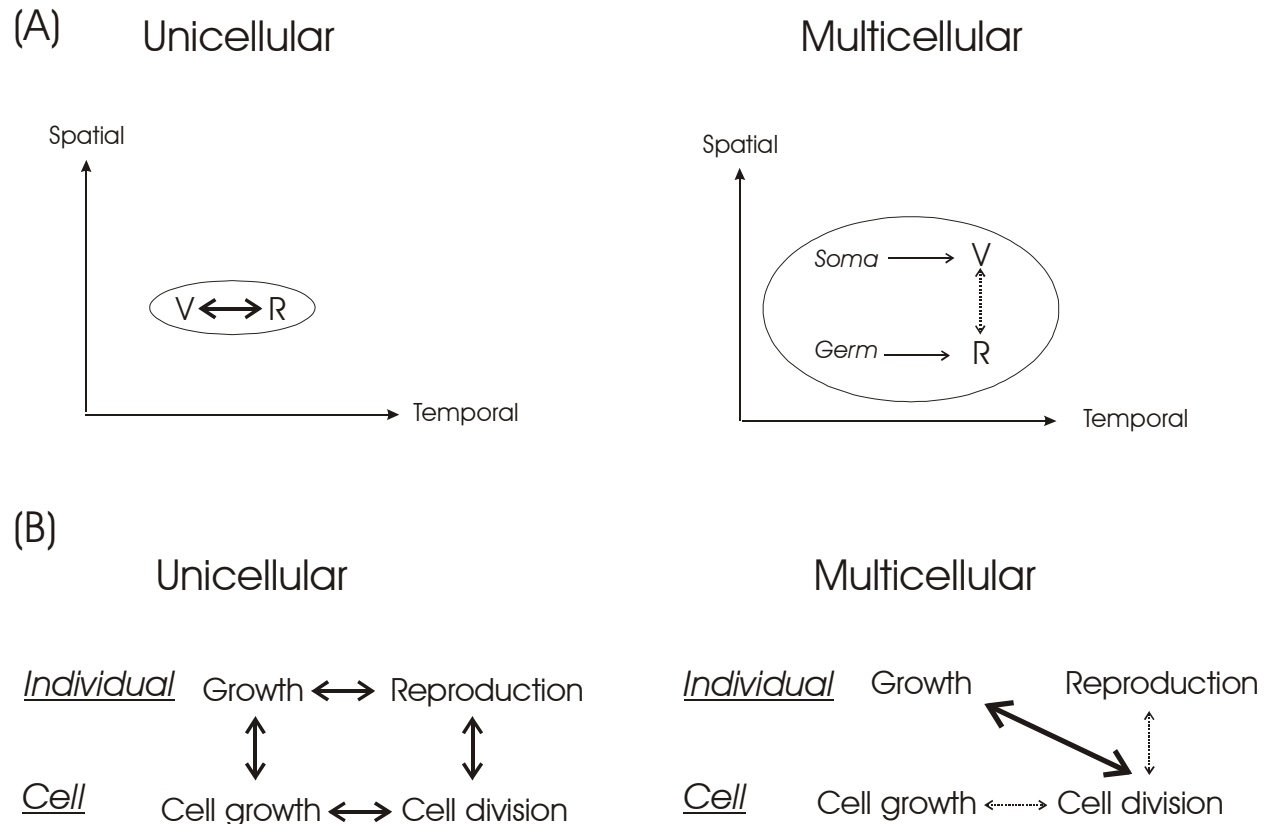
(B) Unicellular — Multicellular

Figure 2. Relationships between vegetative (V) and reproductive (R) functions, on the one hand, and spatial and temporal contexts, on the other hand (panel A), in unicellular versus multicellular individuals, and between processes and properties at the level of the cell and the individual, respectively (panel B); broken arrows denote relationships in which the two components are not necessarily dependent on one another.

Immortality and Totipotency

"Immortality" is used here as the capacity to divide indefinitely, and "totipotency" is defined as the ability of a cell, such as zygote or spore, to create a new individual. We use the term "pluripotent" as the ability of a cell lineage to produce cells that can differentiate into all the cell types (but not into a new functional individual); lastly, "multipotency" refers to the potential of one cell to differentiate into more than one cell type.

Immortality and totipotency are basic life-traits. In unicellular forms, they are manifested/expressed in all cells; cells have both the potential to divide indefinitely (i.e., they are potentially immortal) and to create new individuals, either asexually or sexually (i.e., they are

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totipotent). In unicellular individuals, immortality and totipotency are thus coupled at the cell level.

In multicellular individuals, on the other hand, only one or a few cell lineages manifest both

immortality and totipotency; most other cell lineages have only certain degrees and combinations of

potential for cell division and differentiation. For instance, in groups without an early segregated

germ line, the somatic cell lineages are incapable of continuous division or re-differentiation and

thus they have to be replenished from one or a few pluripotent lineages that remain mitotically active

throughout ontogeny, and can also differentiate into germ cells (e.g., the interstitial I-cells in *Hydra*;

Bode 1996) (Figure 3A). In lineages with a germ line that is terminally differentiated in earliest

ontogeny, various degrees of mitotic capacity (approaching immortality in some stem cell lineages)

and/or potential for differentiation are maintained in the many multipotent somatic stem cells (i.e.,

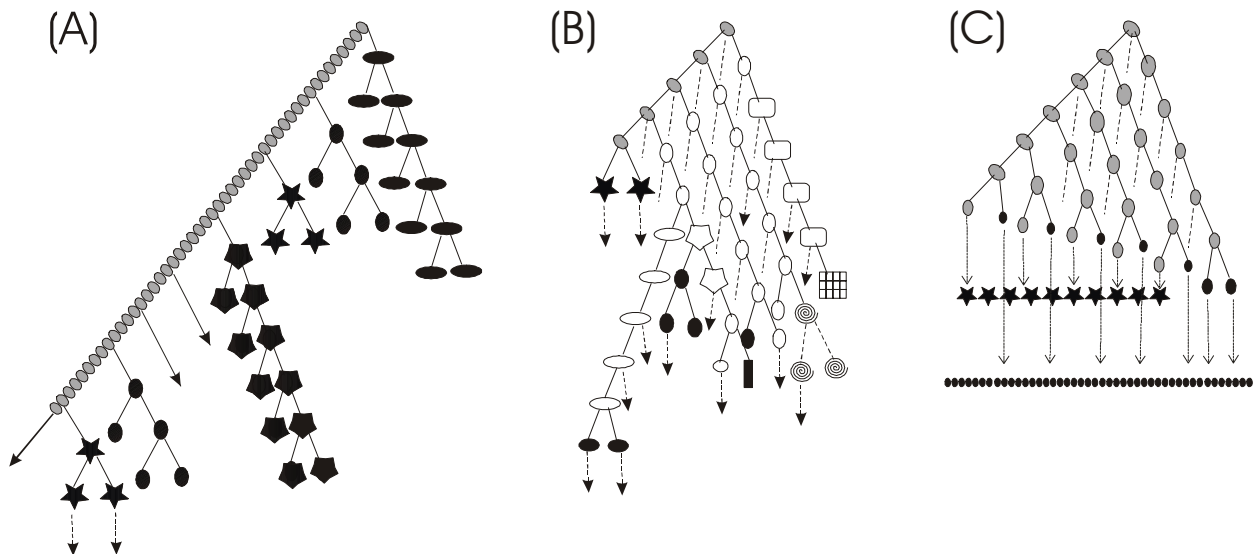secondary somatic differentiation; Buss 1987) (Figure 3B).

Figure 3. The re-organization of immortality and totipotency in three types of development.
Gray ellipses denote totipotent/pluripotent cell lineages; open ellipses mark multipotent cell
lineages; various solid forms indicate different differentiated cell lineages (stars represent the germ
cells, the other shapes represent various somatic cell types); solid-headed arrows indicate post-
embryonic cell divisions in the corresponding cell lineages. (A). The ancestral mode of
development (Buss 1987): a mitotically active and pluripotent lineage gives rise to somatic lineages
(which may or may not divide further), as well as to germ cells throughout ontogeny. (B). The
derived mode of development: a totipotent lineage gives rise to multipotent stem cells (which

produce various cell types during ontogeny) and then differentiates into germ cells early in the development. (C). The *V. carteri* mode of development: a totipotent lineage gives rise to a defined and early-segregated germ line as well as to somatic initials (solid ellipses) that have limited mitotic potential and produce somatic cells with no mitotic and differentiation potential (note the lack of multipotent stem cells, the presence of only one type of somatic cells, and the lack of post-embryonic cell divisions).

(ii) Cellular processes and life-traits

Cell division is an important process in all cellular life-forms. The mechanisms underlying cell division are, however, different between unicellular and multicellular individuals (Fig. 2B). In unicellular individuals, cell division is strictly dependent on cell growth (cells do not divide unless a specific set size is achieved). In many multicellular forms, however, this is not always the case: factors other than cell size (such as intercellular or systemic signals) can trigger cell division. In addition, in unicellular forms cell division is strictly coupled with immortality, whereas in multicellular individuals, cell division has a limited and variable potential in most cell lineages (i.e., they are mortal), and is under the control of the higher-level individual.

(iii) Cellular processes and higher-level functions

Interestingly, cell division and cell growth have different roles at the level of the individual in unicellular compared to multicellular forms (Fig. 2B). In unicellular forms, every cell division results in the reproduction of the individual (cell division is strictly coupled with reproduction). In multicellular forms, cell division is uncoupled from the reproduction of the individual in most cells (i.e., cell divisions do not necessarily result in the reproduction of the higher level). Also, whereas in unicellular forms, cell growth is the main contributor to the growth of the individual (with the exception of extracellular deposits in some lineages), in multicellular forms, the growth of the individual is mostly achieved through increasing the number rather than the size of cells (with some exceptions in lineages where there is significant increase in volume of extracellular matrix, internal space or even cell size).

2. Transition in individuality

We argue here that the unicellular-multicellular transition and the emergence of individuality at a higher level requires: (i) changing the temporal expression of vegetative and reproductive functions into a *spatial context*, (ii) *re-organizing* basic life-traits (such as immortality and totipotency) between and within lower levels, (iii) *de-coupling* processes from one another at the lower level (e.g., cell division from cell growth), (iv) de-coupling certain cellular processes from functions and traits (e.g., cell division from reproduction and immortality) and (v) *co-opting* them for new functions at the higher level (e.g., the co-option of cell division for multicellular growth).

(i) Changing temporal into spatial

During the transition to multicellularity with a germ/soma separation, the expression of vegetative and reproductive functions changes from a temporal to a spatial context (Figure 2A). For instance, in *Chlamydomonas,* the reproductive phase follows the vegetative/cell growth phase and is paralleled by the loss of some of the vegetative functions including motility. In *Volvox*, on the other hand, the *spatial dissociation* of reproductive and vegetative functions between gonidia and somatic cells allows the two sets of functions to take place simultaneously; this is very important in these algae in which the flagellar constraint sets a strong trade-off between reproduction and vegetative functions.

(ii) Re-organizing immortality and totipotency

During the transition to multicellularity, and the emergence of individuality at the higher level, immortality and totipotency become restricted to one or a few specific cell lineages, namely those involved in the reproduction of the higher level. However, many cell lineages maintain various degrees and combinations of mitotic and differentiation potential. This requires the re-organization (i.e., the differential expression) of these traits both among cell lineages and within a cell lineage. As discussed earlier, this re-organization has been achieved differently among the extant multicellular

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groups (Figure 3).

In *V. carteri*, immortality and totipotency are restricted to the zygote (if after a sexual cycle; not shown), or the asexual spore (i.e., gonidia, *a* in Figure 4), the 16 cells following the first 4 embryonic cell divisions (*b*, in Figure 4), and the 16 germ-line precursors (*c* in Figure 4) (Kirk 1994; Kirk et al. 1993). Both traits are lost in one-half of the 32-celled spheroid (*d* in Figure 4), as well as in the small cells (i.e., somatic initials) formed during the asymmetric divisions that take place in the germ line precursor lineage (*e* in Figure 4). The 16 large cells produced by the first asymmetric division of the germ line precursors (i.e., the germ-line blastomeres; *f* in Figure 4) go on and divide asymmetrically for another two or three times (each time renewing themselves and producing a somatic initial) and arrest mitosis two or three cell division cycles before the somatic blastomeres do. These 16 cells (*g* in Figure 4) will differentiate into the germ cells of the next generation. After a total of 11-12 cell divisions, the somatic initials stop dividing and differentiate into somatic cells (*h* in Figure 4), which have no mitotic or differentiation potential (they are terminally differentiated).

It is interesting that in *Volvox*, although immortality and totipotency have become fully restricted to the germ line (and reproduction and individuality at the higher level emerged), somatic lineages have no mitotic or differentiation potential. The two traits have been re-organized between germ and soma, but not within somatic cell lineages. The two sets of traits are still very linked in *V. carteri*; they are either both fully expressed (in gonidia) or both suppressed (in somatic cells). Noteworthy, the sequestration of the germ line was achieved without the evolution of secondary somatic differentiation processes (Figure 3C). This is rather surprising, because it has been suggested that the evolution of an early-defined germ line was possible because, due to the evolution of the multipotent stem cells and secondary somatic differentiation, the ancestral pluripotent germinative lineage was released from the task of producing the somatic tissues and was able to terminally differentiate into germ cells early in development (Buss 1987).
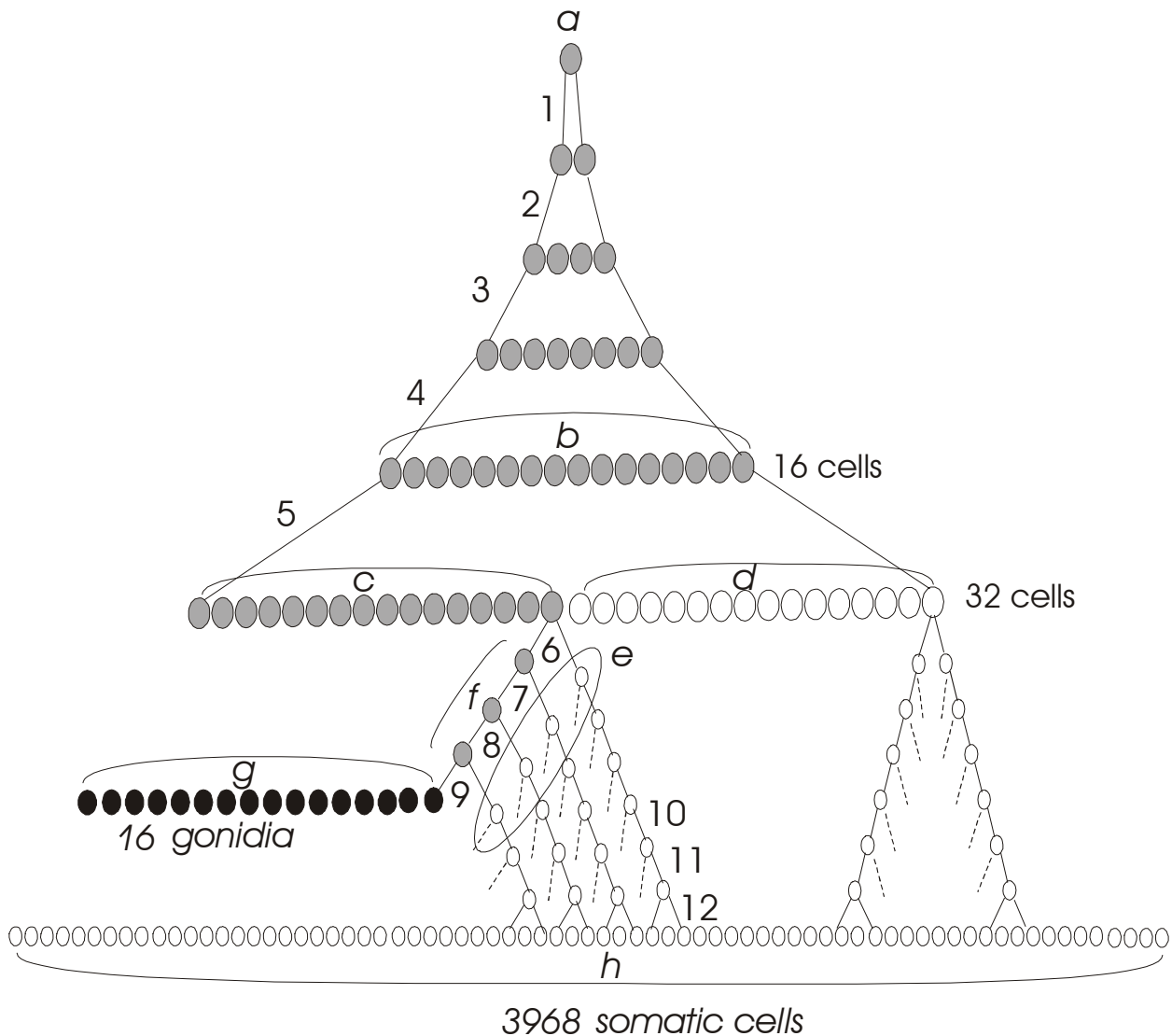
Figure 4. Schematic representation of the development and germ/soma separation in an asexual *V. carteri*. Gray ellipses denote the totipotent and multipotent cell lineages: the asexual spore, i.e., gonidia ("*a*"), the 16 totipotent blastomeres ("*b*"), the germ-line precursors ("*c*"), and the germ-line blastomeres ("*f*"); white ellipses indicate unipotent (i.e., the somatic blastomeres and initials, "*d*" and "*e*") and terminally differentiated somatic cells ("*h*"), and black ellipses indicate terminally differentiated reproductive cells, i.e., gonidia ("*g*"). Note somatic cells ("*h*") have two distinct origins, from germ-line blastomeres ("*f*") via asymmetric divisions and from somatic blastomeres ("*f*") via symmetric divisions. Numbers mark the succession of cell divisions in the embryo. Cells are not represented at scale ("*a*" is ca. 2^9-fold larger than "*g*", and there is a ½-reduction in cell size with every symmetric cell division); all divisions take place under the mother cell wall, in a rather rapid fashion without intervening growth (i.e., palintomy and multiple fission).

(iii) De-coupling cell division from cell growth

To ensure the functionality of the soma, factors other than cell size must be used to determine

which cells divide, when, and how often. This requirement necessitates de-coupling cell division

from cell growth; consequently, a better and more finely tuned control on the replicative potential of

the lower level can be achieved. However, this has not been accomplished in *V. carteri*; cell

division is still strictly dependent on cell growth; reproductive cells have to increase 2^{12} fold in

volume before dividing 12 times to produce the final number of cells in the multicellular individual.

(iv) De-coupling cell division from cell reproduction

To ensure the reproduction of a cell-group (and the heritability of the group traits), cell

division has to be un-coupled from cell reproduction (i.e., the reproduction of the previously

independent unicellular individual) and be co-opted for the reproduction of the higher level (the

group). The ability to reproduce the group can be achieved either by all or only some members of the

group.

The case in which all cells have higher-level reproductive capabilities is best exemplified by a

reproductive mode called autocolony, in which when the group/colony enters the reproductive

phase, each cell within the colony produces a new colony similar to the one to which it belongs; cell

division no longer produces unicellular individuals but multicellular groups. This mode of

reproduction characterizes the volvocacean green algae without a germ/soma separation, such as

Gonium and *Eudorina*.

In *Eudorina*, all cells go through a vegetative and reproductive phase (i.e., divide and produce

each a 32-celled embryo). However, cell division does not produce anymore a number of free

unicellular individuals (such as in *Chlamydomonas*), but rather a new group; cell division has been

thus de-coupled from cell reproduction and has been coupled with the reproduction of the group in

all members of the group. Nevertheless, cell division is still strictly dependent on cell growth: each

cell will start dividing only after a 2^5-fold increase in size was attained, and once cell divisions are

initiated they will continue synchronously until 32 new embryos are formed. Although the stability,

heritability and the reproduction of the higher level are ensured in this way, its individuality is not;

because every member can be separated from the group, live independently and create a new group, such a group is not the smallest physiological and reproductive autonomous unit, thus is not a true individual (in the sense used here).

The case in which only some cells have higher-level reproductive capabilities characterizes lineages with a separation between germ and soma. To achieve this, the coupling between cell division and reproduction is broken in most cells, namely the somatic cells; they reproduce neither themselves (as former free-living unicellular individuals) nor the higher-level unit; cell division is de-coupled from the reproduction of both the lower and higher levels. In this way, somatic cells loose their individuality as well as the right to participate in the next generation; but in doing so they contribute not only to the emergence of individuality at the higher level but also to the emergence of a new level of organization, the multicellular soma. Soma is thus the expected consequence of uncoupling cell division from reproduction in order to achieve individuality at the higher level. *V. carteri* follows this pathway; however, the way in which germ/soma separation was achieved is rather unique among multicellular forms (discussed later).

(v) Co-opting cell division for growth at the higher level

By de-coupling cell division from reproduction, this very important process became available for new functions. We suggest that this event was paralleled by the co-optation of cell division for a new function at the higher level, namely the growth of the multicellular individual. Later, the use of cell division for more than cell multiplication, (i.e., which "gives rise to more entities of the same kind"; Szathmáry and Maynard Smith 1997) may have provided the multicellular lineages with an additional advantage, namely cell differentiation; indeed, in many multicellular lineages asymmetric cell divisions are involved in cell differentiation.

In *Chlamydomonas*, as in other unicellular individuals, cell division is coupled with the reproduction of the individual. Interestingly, in *Volvox,* although the coupling between cell division

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and the reproduction has been broken in the somatic cells, cell division was not co-opted for the post-embryonic growth of the higher-level individual; rather, cell division was simply turned-off in somatic cells. The somatic cells lack the ability to divide post-embryonically; all the cell divisions responsible for the final number of cells in the adult take place during embryonic development (the further growth of the young spheroid is accomplished only through small increases in cell size and through a massive deposition of extracellular matrix). The implications of this outcome are multiple and profound. A direct implication is that soma in *Volvox* differs from the soma of most multicellular organisms. Because somatic cells do not divide, further growth and/or regeneration of the individual are not possible during ontogeny; in addition, because the somatic cells undergo senescence and genetically programmed cell death at the age of 5 days (Pommerville and Kochert 1981; Pommerville and Kochert 1982), the life span of the higher-level individual is limited to the life span of the lower-level somatic cell. Furthermore, although asymmetric cell divisions are involved in the differentiation of somatic cells, the way this process is achieved precludes further re-differentiation. The evolutionary implications of this aspect are discussed next.

IV. Evolvability, genotype-phenotype maps and modularity during the transition in individuality in Volvox carteri

The transition to multicellularity has occurred multiple times in the evolutionary history of life; in addition, it has generally been followed by an increase in diversity and complexity. Nevertheless, although (i) green algae (the charophytes, in particular) are the closest relatives of the more complex land plants, and (ii) 50-75 million years have presumably passed since the divergence of *Volvox* from its unicellular *Chlamydomonas*-like ancestors, none of the *Volvox* lineages appears to have attained high levels of complexity in spite of the multiple events that gave rise to multicellularity and germ/soma separation in this group. What are the reasons for this apparent "slow down" in the evolutionary potential of this group? Could they be traced back to the events associated with the

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transition to multicellularity and the emergence of individuality at the higher level? In *Volvox*, the genotype-phenotype map that emerged during the transition to multicellularity reveals some aspects that might be of relevance to the evolvability of the lineage. We suggest that the way in which the lower-level genotype-phenotype maps are re-organized at the higher level can influence the potential for evolution of the newly emerged multi-level system.

1. Insights from mutant forms

Numerous mutant forms have been described in *V. carteri* (see (Kirk 1998) for a review); in some forms, features that emerged during the transition to multicellularity, mainly individuality and germ/soma separation at the higher level, are affected. Two types of mutants are of relevance in this context, and they provide us with invaluable insight into how the transition to multicellularity has been achieved in this lineage.

In the somatic regenerator mutants, or Reg mutants, the somatic cells start out as small flagellated cells and then enlarge, loose flagella and re-differentiate into gonidia. A number of 39 mutants in four phenotypic classes have been investigated, and all had mutations at the same locus, *regA* (Huskey and Griffin 1979). The gene affected in these mutants has been shown to encode for an active repressor (Kirk et al. 1999) that targets a number of at least 13 nuclear genes whose products are required for chloroplast biogenesis (Choi et al. 1996; Meissner et al. 1999). This finding suggests that the mechanism for the establishment of a stable germ/soma separation in *V. carteri* is based on preventing the somatic cells from growing enough to trigger cell division (by repressing chloroplast biogenesis in these cells; Meissner et al. 1999).

In another class of mutants, the Gls/Reg mutants (Huskey and Griffin 1979), all the cells (though far fewer than in the wild-type, i.e., no more than 128 or 256) act first as somatic cells and then re-differentiate into reproductive cells. These mutants are a reversal to the ancestral *Eudorina*-like type of organization and represent a step back both in terms of complexity (there is no germ-soma separation in these forms) and individuality (each cell will produce a new colony; these mutants are "divisible", thus they are not true individuals). The *Gls* mutation has been mapped to a

gene, *glsA*, which encodes a protein required for the asymmetric divisions responsible for the segregation of germ-line blastomeres and somatic initials (*f* and *e*, respectively, in Figure 4) (Miller and Kirk 1999). Consequently, all cells are equal both in size and potential for differentiation and undergo the ancestral *Chlamydomonas*-like pathway of acting first as vegetative and then as reproductive cells (Tam and Kirk 1991); it should be mentioned that this mutation is only recovered on a *regA* background that allows the growth and thus differentiation of somatic cells into reproductive cells.

In Reg mutants, both immortality and totipotency are re-gained by the somatic cells, and these cells "join" the germ line in participating in the next generation. On the other hand, cells in the Gls/Reg mutants never loose either immortality or totipotency. In neither of these mutants are the two traits expressed partially (e.g., limited mitotic capacity or multipotency). Furthermore, it is interesting that somatic mutant cells in which immortality is re-gained but not totipotency (analogous to the "cancer-like" mutant cells in various other multicellular lineages) are missing in *Volvox,* suggesting that immortality and totipotency are still strongly linked at the lower level in this lineage.

2. Genotype-phenotype maps

During the unicellular-multicellular transition, a new genotype-phenotype map has to be created to reflect the emergence of individuality at the higher level. It is rather intriguing that in *V. carteri*, immortality can be re-gained and individuality can be destroyed by single mutations (such as in *regA* and *glsA*). In other multicellular lineages, such as humans, multiple mutations (each of which requires a minimum of 20-30 cell divisions) are required for immortality (i.e., cancer cells) to be re-gained (e.g., Wright and Shay 2001). The fact that single mutations have such large effects on individuality traits suggests that in *V. carteri*, the genotype-phenotype map at the higher level has been realized through a rather small number of genetic changes. Any attempt to increase the evolvability of these lineages has to first affect the current genotype-phenotype map to allow increased variability of the traits associated with immortality and totipotency (so as to de-couple

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them in the somatic cells) without affecting the individuality of the system (e.g., by evolving mechanisms to control these traits independently, thereby allowing cell replication and/or differentiation in the soma). In other words, the genotype-phenotype map has to at first become more robust (so that small genetic changes will not lead to the re-creation of the maps associated with the previously independent lower levels, as it is currently the case) but flexible (so as to allow improvement through mutation and selection).

To gain such properties a number of small-effect mutations, in a very precise order (such that the viability of the individual under selection is not affected) is required. However, the way in which cell division, cell growth, immortality and potency have been re-organized in *Volvox*, as well as the way the genotype-phenotype map has been created at the higher level, makes the evolution of such traits more difficult. For example, the fact that (i) the de-coupling of cell division from reproduction in somatic cells was not achieved by inventing new ways to control cell division, but rather by blocking it altogether, and (ii) the suppression of cell division was not achieved through evolving some new mechanisms but rather through inhibiting the growth of the cell, strongly limits the evolution of traits that are dependent on these processes. These important complex sets of processes have not been de-coupled from one another through their dissociation at the lower level and their co-option for new functions at the higher level, but rather through the suppression of some of the processes at the lower level (see discussion below); in this way, processes such as cell growth, cell division, and differentiation are not represented in the higher-level map and thus cannot contribute to phenotypic variability.

Improvement is expected to come from mutations that, for instance, allow the somatic cells to regain controlled mitotic activity and some degree of differentiation potential during ontogeny. To achieve this, the multiple fission type of division should be replaced by a binary type, such that cell divisions during adulthood do not result in the duplication of the entire organism (as they do in the *V. carteri* mutants in which somatic cells regain mitotic capabilities); in addition, a binary type of cell division would allow a more finely tuned increase in size, via small increments. In this way,

more phenotypic variability can be achieved and become available for selection. It should be mentioned that the multiple fission type of division is a derived trait, which is thought to have evolved through the modification of the cell cycle via very conserved type of proteins involved in the key pathway that controls both cell division and differentiation in animal cells, namely, the retinoblastoma (RB) family of tumor suppressors (e.g., Sage et al. 2000). Mutations of this gene in *Chlamydomonas reinhardtii* result in the initiation of the cell cycle at a below-normal size, followed by an increased number of cell divisions (Umen and Goodenough 2001). Such an alteration of the cell cycle might have been involved in the evolution of the multiple fission type of cell division, which is considered a pre-condition for the origin of multicellularity in *Volvox* (Kirk 1998). If this is the case, it would argue for another example of achieving an important trait at the higher level (i.e., multicellularity) through a small number of genetic changes, and thus for the potential instability/inflexibleness of the higher-level genotype-phenotype map emerged in this way.

Properties that reduce constraints on change are thought to be very important for the evolvability of a system by conferring flexibility and robustness on processes, and consequently increasing non-lethal phenotypic variation and evolvability (Kirschner and Gerhart 1998). Among these, weak linkage contributes greatly to constraint reduction; in *V. carteri*, however, the linkage (i.e., the dependence of one process on another) between some processes is still very strong, which increases the constraint on change, decreases the potential for phenotypic variation unevenly among the organism's activities, and reduces the evolvability of the lineage.

3. "Divide and rule": Dissociate and control

The fact that in unicellular forms cell division does not occur in the absence of cell growth, and growth to a set size unconditionally triggers cell division, suggests that in these lineages the two processes are part of a single functional module, or two strongly linked modules that are dissociated in time during the life cycle of the individual. Likewise, the vegetative and reproductive functions are realized by the same cell (i.e., they are associated in space), and the latter is dependent on the former (i.e., they are coupled); however, they cannot take place simultaneously (they are dissociated

in time). The dissociation in time of these processes can be seen as analogous to the dissociation in timing of specific modular interactions during the development of complex multicellular organisms.

In contrast, distinct cells types perform these two main functions in multicellular forms with germ/soma separation; the two sets of functions are thus dissociated in space, among different cell lineages, and they can be realized simultaneously. We suggest that during the transition to multicellularity, the translation of the temporal dissociation of certain processes and functions into a spatial one can be accomplished via the dissociation, recombination and redeployment of modules or domains associated with these functions in a spatial (rather than temporal) context at the higher level. In other words, during the unicellular-multicellular transition, the ancestral temporal linkages, such as cell growth-cell division, cell division-reproduction, and growth-reproduction, have to be broken and these processes/functions re-organized in a spatial context. For instance, in some cells (i.e., the somatic cells) the domain associated with cell division has been de-coupled from cell reproduction and was recombined into a new functional module at the higher level, namely that associated with multicellular growth (in somatic cells, cell division shares now the most pleiotropic interactions with somatic growth; the two domains are linked to each other more closely than they are with other modules). In other cells (e.g., germ line) cell division has been de-coupled from cell reproduction but co-opted for reproduction at the higher level. In this way, multicellular growth and reproduction become dissociated in space rather than in time. Likewise, the de-coupling of the modules associated with vegetative and reproductive functions between soma and germ allows for the two sets of functions to be dissociated in space and thus to be achieved simultaneously and independently (not successively as in unicellular forms). The other two integrated domains of the ancestral module, namely cell growth and cell division, have also been dissociated; in a multicellular organism, cell division and cell growth are not necessarily dependent on one another. Furthermore, cell division (i.e., asymmetric cell division) has been redeployed in the context of a multicellular organism with distinct cell types, and has been co-opted for cell differentiation.

The modular domains associated with the two complex sets of traits, immortality and

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totipotency, appear to have also been dissociated and re-combined/redeployed during the transition to multicellularity. The domains associated with immortality and totipotency have been themselves further dissociated into sub-domains, such that the potential for cell division and differentiation can be controlled and expressed outside the context of immortality and totipotency, respectively. Somatic cell lineages can be either mitotically active throughout ontogeny (e.g., some stem cell lineages) or can maintain only a reduced (and/or pre-set) mitotic potential, with or without any potential for differentiation; if they do have such potential, they can differentiate in one or more cell types, depending on the type of cell lineage. Therefore, interactions between modular domains associated with immortality and totipotency have been spatially dissociated in a multicellular organism, both between germ (in which immortality and totipotency are still coupled) and soma (in which the two traits are un-coupled), as well as among the various somatic cell lineages which can enjoy distinct combinations and degrees of replicative and differentiation potential.

4. The Gordian knot and evolvability in Volvox

How have the domains and modules associated with cell division, cell growth, immortality, and totipotency become re-organized during the transition to multicellularity in *Volvox*? It is interesting that a single mutation, in the *regA* gene, results in the expression of reproductive traits (both immortality and totipotency) in the somatic cells; thus, *regA* can be seen as a master regulatory gene for reproduction, analogous, for example, to the master control for the complex formation of the eye, *eyeless,* in *Drosophila* (Halder et al. 1995), and the two sets of traits as part of the same module. Furthermore, it is noteworthy that *regA* manifests its effect on the reproduction of the individual indirectly, by suppressing cell growth, which in turns blocks cell division in somatic cells. Therefore, because a single gene, *regA*, affects both cell growth and division in *V. carteri* argues for the two processes being associated with domains of the same genetic module with strong pleiotropic effects within.

Thus, although the switch from a temporal to a spatial dissociation of certain domains has been accomplished in *V. carteri*, and a germ line (with immortality and totipotency) and a soma have

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evolved, the re-organization of the domains associated with these complex sets of traits at the higher level was achieved in a rather peculiar way. *Volvox* was not able to dissociate and control (i.e., differentially express) the ancestral module associated with immortality and totipotency in somatic cells; instead, in *Volvox,* both domains are entirely suppressed in the somatic cells. Furthermore, the suppression of both domains was achieved by acting on a single process, namely cell division. Moreover, the way in which *Volvox* suppressed cell division was not by acting directly on the domain associated with the mitotic potential of the cells but rather indirectly by acting on a domain that was still very linked to it, that is that associated with the growth of the cell. By suppressing cell growth in somatic cells, cell division is repressed and the potential for gaining immortality and totipotency is "under control"; however, this type of "ultimate" control later interfered with the potential for evolution in this lineage (discussed below).

The mechanism that is responsible for germ/soma differentiation in *V. carteri* reveals another peculiar way of ensuring the emergence of reproduction at the higher level. Although cell differentiation involves asymmetric cell divisions, they are not involved in the differential segregation of germ-line factors (such as the P granules in the nematode *Caenorhabditis elegans*; e.g., (Seydoux and Schedl 2001)); rather, asymmetric divisions ensure that the gonidia-precursors remain large enough such that the capacity to grow and further divide is not lost (as it is in the somatic precursor cells, due to the expression of *regA*). Thus, the way in which asymmetric cell divisions determines the cell fate of the somatic cells is by acting on the ancestral cell growth-cell division linkage; it does not involve new mechanisms or new pathways of gene regulation. We suggest that the developmental path observed in *V. carteri* is a consequence of its "inability" to dissociate lower-level modules and recombine/redeploy certain domains into new functional modules at the higher level; as an alternative strategy to ensure and maintain individuality at the higher level, *V. carteri* entirely suppressed domains of some of these modules at the lower level, including cell growth and cell division. The knot that could not be untied was cut: a difficult problem was solved by a quick and decisive action. In this way, the risk of regaining immortality

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and totipotency at the lower level (as exemplified by the somatic regenerator mutants) was somewhat avoided; but so were other processes, including post-embryonic growth and cell differentiation. By completely suppressing the domains associated with cell growth and cell division in the somatic cells, certain sets of processes/trait were not recombined or re-deployed in the new context, and co-opted for new functions at the higher level. Unfortunately, these traits proved to be important for the evolutionary adaptability of a multicellular lineage. Without them, *Volvox* did not and will not easily attain higher levels of complexity. Due to its unique type of soma, *Volvox* is missing more than the ability to grow, regenerate, or live longer (whose lack evidently does not constitute strong disadvantages in the environment to which these algae are adapted, namely temporal aquatic habitats).

An important evolutionary consequence of modularity is allometry; this occurs when different parts of the body grow at different rates. Allometry can generate evolutionary novelty by small, incremental changes that eventually can cross developmental thresholds; a change in quantity can become a change in quality (e.g., Brylski and Hall 1988). Under the constraint of multiple fission and palintomy, the body parts in *V. carteri* grow at the same rate, so the potential for generating novel traits in this way is not possible in this lineage. In addition, without a mitotically active multipotent stem cell lineage or secondary somatic differentiation there is less potential for cell differentiation and further increase in complexity.

Volvox managed to dissociate the vegetative functions (motility in particular) from the reproduction of the multicellular individual such that both selective advantages, namely, large size and mobility are achieved. However, although the solution found provides the lineage with the immediate increase in fitness, it affected the potential for modularity to participate in further altering developmental processes to increase the evolutionary adaptability of the lineage. Thus, evolutionary modularity was traded off for functional modularity. The inability to dissociate some of the domains of the lower-level modules might reflect in the developmental constraints and the low degree of freedom of the phenotype in this lineage, especially with respect to body size (Koufopanou and Bell

1991).

V. Concluding remarks

The transition to multicellularity has happened numerous times in the evolutionary history of eukaryotes; of some 23 protist groups, 17 have multicellular representatives (Buss 1987). However, only three major groups, namely, fungi, animals and plants, have achieved high levels of complexity. In addition, the extant groups appear to vary in their levels of diversity, suggesting distinct potentials for evolutionary adaptability, or evolvability. Various processes, such as modularity, robustness to genetic variability (Conrad 1990), robustness to developmental variation (Kirschner and Gerhart 1998), and heritability of fitness (Michod 1999; Michod et al. 2002), play important roles in evolvability. Here, we suggest that some of these processes gain new dimensions in the context of evolutionary transitions in individuality, and that the potential for further evolutionary adaptability of a lineage might be at some extent influenced by the way that the transition in individuality has been achieved.

A new genotype-phenotype map has to be created at the newly emerged higher level through the re-organization of the genotype-phenotype maps of the previously independent lower levels. Modularity plays a crucial role in this process; *the way in which modules become dissociated at the lower level and recombined or redeployed at the higher level reflects in the flexibility and robustness of the newly emerged higher-level genotype-phenotype map*. Some modules are more resistant to dissociation than others; if un-dissociable, their domains might not be represented in the genotype-phenotype of the higher level and thus cannot contribute to phenotypic variability. Moreover, the strong linkage between modules at the lower level can reflect in developmental constraints at the higher level.

The differential expression of immortality and totipotency traits between cell lineages and among phylogenetic groups is reflected in the various developmental programs in the extant multicellular lineages. The co-option of cell division for growth and cell differentiation at the higher level sets the premises for the evolution of soma and increase in complexity in lineages with a

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germ/soma separation. Likewise, the de-coupling of cell division from cell growth allowed a better control of the replicative potential at the lower level, and thus a better functionality of the higher level. Selfish mutants that occur at the lower level and threaten the individuality of the higher-level might be indicative of the way in which individuality has been achieved in a particular lineage, as well as of the way that modules have been dissociated and certain domains co-opted for new functions at the higher level. Lastly, the diversity in developmental types and complexity levels among multicellular lineages might represent outcomes of distinct strategies to reach "good solutions" to various problems associated with the transition in individuality. The specific paths, however, can interfere with the potential for further evolution of a lineage. *Differences in evolvability among lineages might therefore be traced back to early events associated with the transition in individuality.*

Summary

During evolutionary transitions in the units of evolution, individuality emerges at a new and higher level. Here, we argue that *the transition from unicellular to multicellular organisms requires the re-organization at the higher level of certain basic life properties, such as growth, reproduction, immortality and totipotency, as well as of the cellular processes associated with them (e.g., cell division and cell growth).* Furthermore, we suggest that the way in which this re-organization is achieved is not only instrumental for the emergence of individuality at a higher level but can also affect the potential for evolution, i.e., evolvability, of the newly emerged higher-level unit. We use the volvocalean green algal group to argue that during the unicellular-multicellular transition: (i) fundamental processes and functional modules have to be dissociated at the lower level and recombined or redeployed to ensure the emergence of individuality and new functions at the higher level; (ii) although modularity is generally expected to improve evolvability, during transitions in individuality this expectation is complicated and sometimes compromised by constraints at the lower level; (iii) the way in which complex sets of traits (and the genotype-phenotype maps associated with them) are re-organized during the transition in individuality affects the flexibility and

robustness of the new genotype-phenotype map which emerges at the higher level, and can interfere with the potential for further evolution of the lineage. We think that the unique way in which cell division, cell growth, immortality and totipotency have been re-organized in the multicellular green alga *Volvox carteri*, as well as the way in which a new genotype-phenotype map has been created at the higher level, limited the evolvability of this lineage.

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