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Cooperation and conflict in the evolution of individuality IV. Conflict mediation and evolvability in *Volvox carteri*

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Abstract

The continued well being of evolutionary individuals (units of selection and evolution) depends upon their evolvability, that is their capacity to generate and evolve adaptations at their level of organization, as well as their longer term capacity for diversifying into more complex evolutionary forms. During a transition from a lower- to higher-level individual, such as the transition between unicellular and multicellular organisms, the evolvability of the lower-level (cells) must be restricted, while the evolvability of the new higher-level unit (multicellular organism) must be enhanced. For these reasons, understanding the factors leading to an evolutionary transition should help us to understand the factors underlying the emergence of evolvability of a new evolutionary unit. Cooperation among lower-level units is fundamental to the origin of new functions in the higher-level unit. Cooperation can produce a new more complex evolutionary unit, with the requisite properties of heritable fitness variations, because cooperation trades fitness from a lower-level (the costs of cooperation) to the higher-level (the benefits for the group). For this reason, the evolution of cooperative interactions helps us to understand the origin of new and higher-levels of fitness and organization. As cooperation creates a new level of fitness, it also creates the opportunity for conflict between levels of selection, as deleterious mutants with differing effects at the two levels arise and spread. This conflict can interfere with the evolvability of the higher-level unit, since the lower and higher-levels of selection will often "disagree" on what adaptations are most beneficial to their respective interests. Mediation of this conflict is essential to the emergence of the new evolutionary unit and to its continued evolvability. As an example, we consider the transition from unicellular to multicellular organisms and study the evolution of an early-sequestered germ-line in terms of its role in mediating conflict between the two levels of selection, the cell and the cell group. We apply our theoretical framework to the evolution of germ/soma differentiation in the green algal group Volvocales. In the most complex member of the group, *Volvox carteri*, the potential conflicts among lower-level cells as to the "right" to reproduce the higher-level individual (i.e. the colony) have been mediated by restricting immortality and totipotency to the germ-line. However, this mediation, and the evolution of an early segregated germ-line, was achieved by suppressing mitotic and differentiation capabilities in all post-embryonic cells. By handicapping the soma in this way, individuality is ensured, but the solution has affected the long-term evolvability of this lineage. We think that although conflict mediation is pivotal to the emergence of individuality at the higher-level, the way in which the mediation is achieved can greatly affect the longer-term evolvability of the lineage.

Keywords: Levels of selection; Germ-line; Mutation; Green algae; Altruism; Group selection

1. Evolvability, evolutionary transitions and fitness

The continued well being of evolutionary individuals depends upon their evolvability, which, in our

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view, includes not only their capacity to generate and evolve adaptations, but also as their capacity to diversify into more complex evolutionary units. To adapt evolutionary individuals must be units of selection, and, ever since Darwin, we have understood that a unit of selection must possess the following properties: the struggle to survive; variation; and, heritability, so that offspring resemble parents. We may combine these three properties and simply say that a unit of selection must have heritable variation in fitness. There are a variety of such units in biology: genes, gene networks, prokaryote cells, eukaryotic cells (cells in cells), multicellular organisms, and groups and societies. How and why did these different kinds of evolutionary units arise?

In the present paper, we consider our theory for the origin and evolution of new kinds of individuals (Michod, 1996, 1997, 1999; Michod and Roze, 1997, 1999, 2000) from the perspective of evolvability. During a transition to a higher-level individual, such as between unicellular and multicellular organisms, the evolvability of lower-level units (for example, cells) must be restricted, while the evolvability of the new higher-level unit (for example, the multicellular organism) must be enhanced. Consequently, understanding the factors leading to an evolutionary transition should help us to understand the factors underlying evolvability.

We study the role of the process of conflict mediation in enhancing the evolvability of a new unit of selection. As an application of our theory, we consider a general feature of multicellular organisms, the separation and specialization of germ and somatic-lines of cells, and apply our results to the evolution of germ/soma differentiation in the green algal group, Volvocales (Nedelcu and Michod, 2003). We use the terms “germ” and “soma” in the sense of there being two kinds of cells in the multicellular group, cells that are specialized in contributing to the next generation of individuals and cells that are specialized in vegetative functions and do not directly reproduce the next generation of individuals. Even organisms often regarded as not having a germ-line, such as plants, have cells that are specialized in reproductive and vegetative functions, and so meet our criteria of reproductive specialization. Specialization of cells in reproductive and vegetative functions is an almost universal feature of multicellular life.

The basic problem in an evolutionary transition is to understand how a group of individuals becomes a new kind of individual, possessing the properties of heritable variation in fitness at a new level of organization—tantamount to evolvability of the new evolutionary individual. During evolutionary transitions, preexisting individuals form groups, within which interactions occur that affect the fitnesses of both the individuals and the group. For example, under certain conditions, bacteria associate to form a fruiting body, amoebae associate to form a slug, solitary cells form a colonial group, normally solitary wasps breed cooperatively, birds associate to form a colony, and some mammals form societies. In addition, about 2 billion years ago, archaebacteria-like cells (destined to be the ancestors of all eukaryotes) began alliances with other bacteria to form the first eukaryotic like cell.

Such associations and groups may persist and reform with varying likelihood depending on properties of the group and the component individuals. Initially, group fitness is the average of the lower-level individual fitnesses, but as the evolutionary transition proceeds, group fitness becomes decoupled from the fitness of its lower-level components. Indeed, the essence of an evolutionary transition in individuality is that the lower-level individuals must “relinquish” their “claim” to fitness, that is to flourish and multiply, in favor of the new higher-level unit. This transfer of fitness from lower to higher-levels occurs through the evolution of cooperation and mediators of conflict that restrict the opportunity for within-group change and enhance the opportunity for between-group change. Until, eventually, the group becomes a new evolutionary individual in the sense of being evolvable—possessing heritable variation in fitness (at the new level of organization) and being protected from the ravages of within-group change by adaptations that restrict the opportunity for defection (Michod, 1999).

2. A model for the evolution of an early-segregated germ-line

2.1. Conflict mediation

Key in the conversion of a group of cooperators to a new evolutionary individual is the evolution of conflict mediators, genes that tweak development

and/or the ways in which the groups are organized and by so doing tilt selection in favor of the group and away from the level of the cell. Although the underlying mechanisms may be diverse (germ-line, apoptosis, self-policing, mutation rate, determinate growth, reproductive mode and propagule size), conflict mediation serves to enhance the evolvability of the higher-level unit by restricting the evolvability of composite lower-level units. However, as we discover below in the green algal group Volvocales, the way in which conflict mediation is accomplished may be short-sited and end up interfering with the long-term evolvability of the new multicellular unit.

The steps involved in the origin of multicellular life have been discussed by a variety of authors (see, for example, Maynard Smith and Szathmáry, 1995). As mentioned earlier, the initial step was likely the formation of cell-groups leading to some kind of primitive colonial life. These cell groups could have been formed in several ways, such as through aggregation, fragmentation, or single cell spore-like reproduction (Michod and Roze, 2000, 2001; Roze and Michod, 2001). Single cell spore-like reproduction may have been accomplished simply by the failure of the daughter cells to separate following cell division. For example, the multiple fission mode of reproduction in volvocalean green algae discussed later is particularly well suited to forming cell groups in this way. Along with the formation of cell groups, there was likely the evolution of cooperative functions which benefited the group. Cooperative interactions are fundamental to the emergence of new individuals, as only cooperation exports fitness from the lower to the new higher level. However, the evolution of cooperation also sets the stage for conflict. For multicellular organisms to emerge out of cell groups, conflict mediation is needed to regulate the levels of selection conflicts inherent in the initial structure of cell-groups (Michod, 1999). The developmental programs and organizational structures created through conflict mediation are the first emergent functions at the higher level. The evolution of a way of regulating conflict means that the group is no longer a collection of lower-level units, it is on its way to becoming a new higher-level individual. For example, in the case of the evolution of reproductive specialization through a germ and somatic-line of cells (considered here), the group is no longer divisible, because certain cells have special functions.

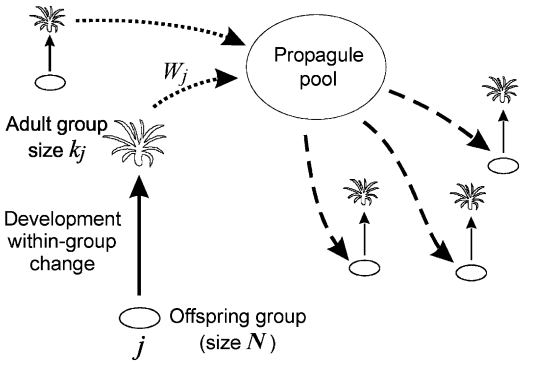

Fig. 1. Multi-level framework for the origin of multicellular organisms. The subscript j refers to the number of cooperating cells in a propagule; $j = 0, 1, 2, \ldots, N$, where N is the total number of cells in the offspring propagule group, assumed constant for simplicity. The variable k_j refers to the total number of cells at the adult stage of propagules that start out with j cooperating cells. The variable W_j is the fitness of group j, defined as the expected number of propagules produced by the group, assumed to depend both on size of the adult group after development and its functionality (or level of cooperation among its component cells) represented by parameter β in the models later.

2.2. Multi-level selection model

A multi-level selection approach begins by partitioning the total change in frequency of genotypes into within and between-group components. Groups are defined by a group property, usually the group frequency of a phenotype or genotype (or some other property reflecting group composition). In our model, we assume that offspring groups are composed of N cells as in Fig. 1 (Michod, 1999; Michod and Roze, 1999, 2000). The level of kinship among cells in the offspring groups is determined by N.

During development, cells proliferate and die (possibly at different rates depending on cell behaviour) to create the adult cell group. Cell behaviour is assumed to be determined by a single haploid genetic locus with two alleles, C (for cooperative cells) and D for defecting cells. Deleterious mutation (from C to D) may occur during cell division leading to the loss of cooperative cell functions (such as the propensity to become motile in a *Volvox* colony considered later) and a decrease in fitness of the adult group. The

adult group produces offspring groups of the next generation. We have previously considered several different modes of reproduction according to how the offspring group is produced: single cell (or spore reproduction), fragmentation and aggregation. In the present model, we consider only single cell reproduction and assume that development starts from a single cell ($N = 1$), as is the case in *Volvox carteri* and the other members of the volvocine lineage (see Fig. 5 later).

The basic parameters of the model include development time, t, the within organism mutation rate from C to D per cell division, μ, the effect of mutation on the cell replication rate, b ($b < 1$ or $b > 1$ means uniformly deleterious or selfish mutations, respectively), the deleterious effect of mutation on the cell group or organism, β, and the propagule size, N. The parameter β measures the benefit of cooperation at the group or organism level, and, hence, the deleterious effect of mutation on group fitness. In addition, there is a parameter that tunes the relative effect of group size on fitness.

To study how evolution may shape development and the opportunity for selection at the two levels of organization, we assume a second modifier locus with two alleles M and m. The m allele encodes the ancestral state (no mediation) while the M allele changes the parameters of development and/or selection at the primary cooperate/defect locus. In this way, we may understand the evolution of developmental programs that permit and enhance the evolvability of the group. The conflict mediator M allele may affect virtually any aspect of the model, such as propagule size, N (studied elsewhere, Michod and Roze, 1999, 2000; Roze and Michod, 2001), adult size (whether it is determinate or indeterminate, Michod and Li unpublished results), self-policing, programmed cell death, development time and whether there is an early-segregated germ-line. To study the evolution of self-policing, we assumed the modifier affects the parameters of selection at both levels, b and β, reducing the temptation to defect at some cost to the group (Michod, 1996; Michod and Roze, 1999). In the case of the evolution of programmed cell death, we assumed the modifier directly decreases the replication rate of mutant cells (Michod and Nedelcu, 2003). In the present paper, we study how the M allele may create an early-segregated germ-line.

2.3. Details of germ-line model

Using the two locus modifier model just outlined, we have studied the evolution of a sequestered germ-line (Michod, 1996; Michod and Roze, 1997, 1999). We assume that the modifier M allele creates two separate lineages of cells within the group, a germ- and somatic-line, which may each undergo a different number of cell divisions and experience a different mutation rate. In our previous work, we assumed that the germ-line was sequestered as a *single* cell set aside during the *first* cell division. Most organisms depart from this ideal, including *V. carteri*, and sequester cells later in development. For this reason, we model the selective forces acting on the time of sequestration, θ, and the number of cells sequestered, ν. In our previous work, we assumed $\theta = \nu = 1$.

A critical assumption concerns the definition of fitness in groups with and without germ/soma differentiation. In groups without separate germ and somatic cells, all cells in the group perform reproductive (germ) and vegetative (somatic) functions. We assume fitness is proportional to group size, the number of cells in the group, k, multiplied by a term representing the functionality of the group, $(1 + \beta f)$, where f is the frequency of cooperative cells in the group and β is the benefit of cooperation discussed previously. This gives Eq. (1) for the fitness of groups without germ/soma differentiation.

$$W = k(1 + \beta f) \tag{1}$$

In the groups with separate germ and somatic cells, there must be fewer cells available for somatic function, because some cells, ν, have been put aside to produce the germ-line cells. Consequently, there must be a cost to sequestering the ν cells; how should we represent this cost during this early stage of germ/soma evolution?

We consider the initial phases of germ/soma evolution in which the presence of a differentiated germ-line is the only difference between the two kinds of groups (groups made up of undifferentiated cells and groups made up of differentiated germ and somatic cells). For example, we assume that the resources available to the group remain essentially unchanged by germ/soma differentiation, except as a result of changes in the numbers of cooperative and mutant cell types. The number of cell types change in

the differentiated group, because cooperative behaviors are expressed only by somatic cells. By virtue of specializing in a single function, such as motility, somatic cells may be able to perform the vegetative functions better or for longer periods of time (after all somatic cells do not have to take time to reproduce the group). These benefits of specialization are clearly important and make it easier to evolve germ/soma differentiation, but we ignore these effects here; their consequences seem obvious (in the sense that they will make it easier to evolve germ/soma differentiation), and our interest lies elsewhere in the mediation of conflict between levels of selection brought about by the evolution of the germ-line.

To understand the cost of sequestering ν cells to create the germ-line, we think of these ν cells in terms of two descendent populations of cells, according to the conditions either of their new existence as differentiated germs, or their prior existence as members of an undifferentiated cell group. In the case of their prior existence, the ν cells would have given rise to a descendent population of size, say, k_ν cells undifferentiated with respect to reproductive or vegetative functions. In the case of their new existence as differentiated germs, the germ sample (the ν cells) replicates for perhaps a different period of time with a different mutation rate, giving rise to a descendent population of size, say, K_ν germ cells. The cost of the germ-line can be seen as stemming from either the new K_ν germ cells or the missing k_ν cells available for vegetative (somatic) functions.

Following the definition of fitness in undifferentiated groups (Eq. (1)), we assume that fitness of the newly differentiated group is fecundity multiplied again by a term representing the functionality of the group (which has now changed because of the germ-line cost). Fecundity is the number of gametes, taken to be proportional to size of the germ-line K_ν. For concreteness, we think of the functionality as the amount of resources accrued by the group for reproduction and we let fitness equal the total number of gametes produced multiplied by the amount of resources received by each gamete. We assume that differentiation does not change the level of resources available to the group, taken to be proportional to the undifferentiated group size, k, except that there are K_ν new germ cells present, or k_ν somatic cells absent (depending on how the germ-line cost is interpreted). When the cost of the germ-line is based on the new germ cells, fitness equals $K_\nu\ (k - K_\nu)(1 + \beta f_\nu)/K_\nu = (k - K_\nu)(1 + \beta f_\nu)$, where f_ν is now the frequency of cooperating cells in the soma (instead of in the total group), giving Eq. (2).

$$W_\nu = (k - K_\nu)(1 + \beta f_\nu) \tag{2}$$

Eqs. (1) and (2) were used previously in our study of germ-line evolution when sequestration of a single cell occurred during the first cell division (Michod, 1996, 1999; Michod and Roze, 1997, 1999, 2000). When the cost of the germ-line is based on the missing somatic cells, k_ν, fitness equals $K_\nu(k - k_\nu)(1 + \beta f_\nu)/K_\nu = (k - k_\nu)(1 + \beta f_\nu)$ giving Eq. (3).

$$W_\nu = (k - k_\nu)(1 + \beta f_\nu) \tag{3}$$

In both Eqs. (2) and (3), the number of gametes cancels and fitness depends only on the amount of resource. In our model, producing many low quality gametes is the same as producing a few high quality ones. As shown later, depending upon how the cost of a germ-line is paid (whether we use Eqs. (2) and (3)), early sequestration of the germ-line may be advantageous or not.

As already mentioned, in many organisms with a germ-line, the germ-line is sequestered not during the first cell division but later in development. For example, in the green alga, *V. carteri* (considered in more detail later), the precursors of the germ-line are formed after five cell divisions, but the germ-line is sequestered only after the ninth cell division. We extend our model to study the time of sequestration by introducing two new parameters defined in Fig. 2, the time when the segregation occurs (θ) and the number of cells sequestrated to form the germ-line (ν). In our previous model, the germ-line was differentiated from the soma at the beginning of the development, from

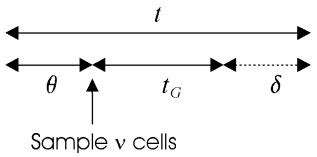

Fig. 2. Time of germ–soma segregation. After θ divisions, ν cells are sampled to form the germ-line, each cell in the germ-line divides t_G more times. The number of cell divisions in the soma is t and $\delta = t - t_G - \theta$ is the difference in number of cell divisions between the germ-line and the soma.

one single cell so that $\theta = 1$, $\nu = 1$ and t_G was a free parameter. Among the sample of cells sequestrated to form the germ-line at time θ, there will be some C cells and some D cells, the number of C cells being given by a probability distribution. The technical details underlying this distribution and its implications for the model are given in the Appendix A.

Using two locus modifier techniques outlined earlier and discussed further in Appendix A, we have studied the evolution of germ-line modifiers that sequester ν cells after θ cell divisions for the two kinds of fitness functions (two kinds of germ-line cost) given in Eqs. (2) and (3). In both formulations, fitness depends upon the resources available to the germ-line, however in Eq. (2), the cost of the germ-line depends upon the number of gametes produced by the germ-line, while in Eq. (3), the cost depends on the missing cells that are no longer available for vegetative function.

3. Results

Results using fitness Eq. (2) are given in Fig. 3 for the following parameter values $\mu = \mu_G = 0.003$, $\beta = 3$, $b = 1.05$, $t = 30$ and no sex or recombination ($r = 0$). These parameter values have been explored

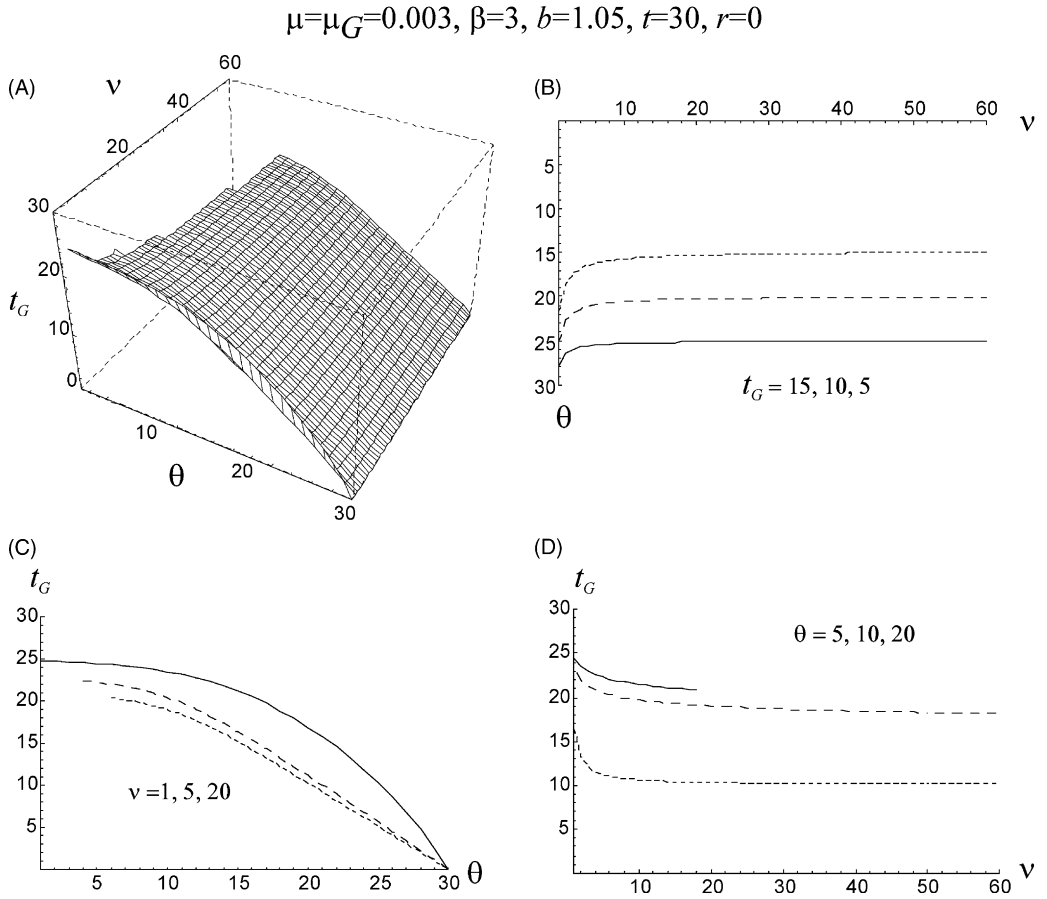

Fig. 3. Evolution of germ-line segregation using fitness Eq. (2). In Eq. (2), we represent the cost of the germ-line by subtracting off the number of resulting germ cells (after the ν cells replicate for t_G times), $k - K_\nu$. The germ-line modifier is characterized by three parameters: the time of sequestration, θ, the number of divisions after sequestration, t_G, and the number of cells sampled, ν. Germ-line evolves for parameter values below the surfaces in all panels. The total number of replications in the germ is $t_G + \theta$. The parameters for the three slices in the two dimensional plots are given from top to bottom. For example, in panel (D), the top solid curve is for $\theta = 5$ and the bottom dotted curve is for $\theta = 20$. Propagule size $N = 1$. Methods used to construct the graphs are given in the Appendix A.

and discussed in our previous work and they are used here for comparison purposes. Please consult our previous papers for discussion of the rationale for using these parameter values (for example, Michod and Roze, 2000). The germ-line modifier evolves for parameter values below the surfaces plotted in Fig. 3. In our previous work, the germ-line modifier was characterized by a single parameter, δ, the difference in number of cell divisions between the germ-line and the somatic-line. However, the germ-line modifier is now characterized by three parameters: the time of sequestration, θ, the number of divisions after sequestration, t_G, and the number of cells sampled, ν. The difference in number of cell divisions between the germ-line and the soma, $\delta = t - t_G - \theta$, now depends on two of the germ-line parameters in addition to the soma parameter, t. The number of cell divisions after segregation of the germ-line, t_G, is important in that it (in conjunction with the number of cells sampled, ν) determines the size of the germ-line and ultimately the number of gametes, that is this parameter determines fecundity (see Fig. 8 later).

The results in Fig. 3 are straight forward; it is easier for a germ-line to evolve (larger germ-lines may be produced) the earlier the germ-line is sequestered (Fig. 3, especially panel (B)), the lower the number of times it divides, and the fewer number of cells that are sampled. This is because there are only advantages to early segregation and low replication (in terms of a lower effective deleterious mutation rate resulting from the fewer number of cell divisions), and the cost of the germ-line is smaller the fewer cells that are

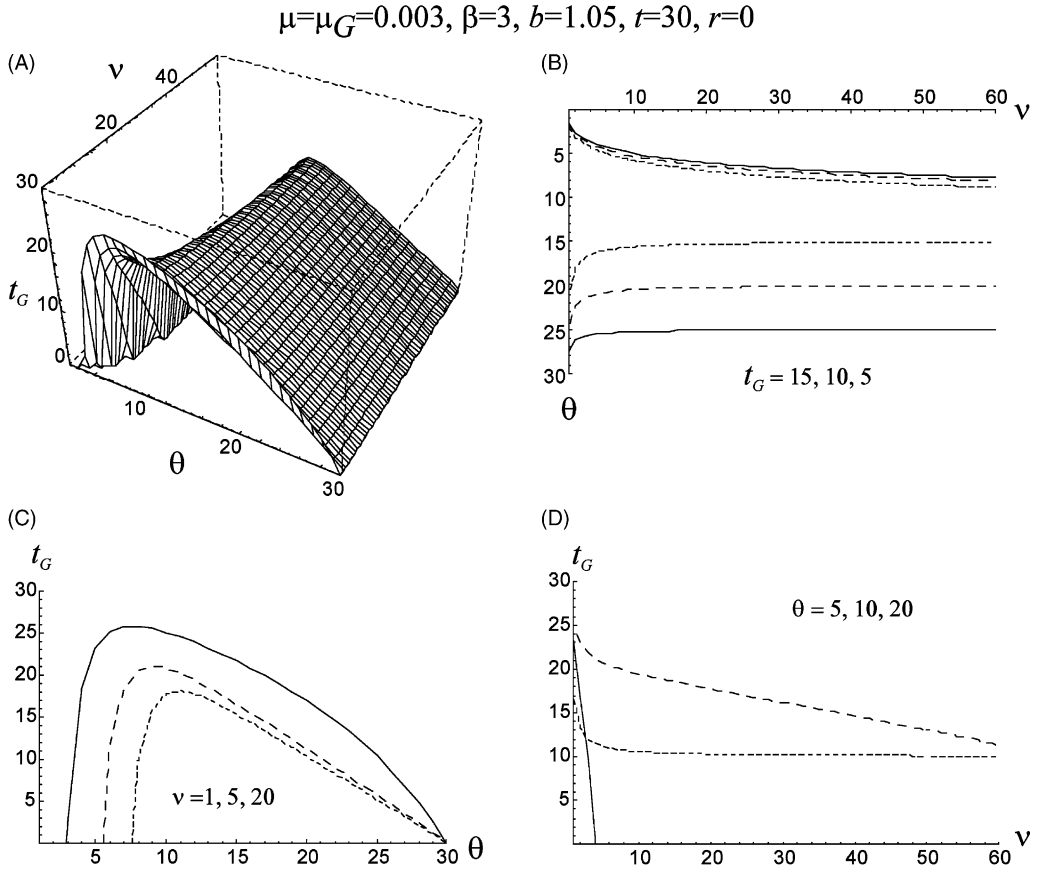

Fig. 4. Evolution of germ-line segregation using fitness Eq. (3). In Eq. (3), we represent the cost of the germ-line by subtracting off the missing somatic cells, $k - k_\nu$. Germ-line evolves for parameter values below the surfaces in all panels. See legend to Fig. 3. Propagule size $N = 1$. Methods used to construct the graphs are given in the Appendix A.

sampled. Recall, that in our formulation, fitness does not depend on the size of the germ-line directly (only through resource availability), since the number of gametes cancels out in the formulation of fitness. Organisms following this model (using Eq. (2)) should form a germ-line by sequestering a single non-dividing cell during the first cell division.

What about when the resources available to the germ-line depend on the number of cells missing unavailable for vegetative (somatic) function? The missing cells are those that would have been formed by the ν cells sequestered to form the germ cells. In Fig. 4, we show the results for the same set of parameter values as used in Fig. 3, except using Eq. (3) instead of Eq. (2). In this case, there is a cost to the germ-line in terms of missing somatic cells, and, so, there is an intermediate optimum time for sequestration, θ (Fig. 4, especially panels (B) and (C)). Smaller θ is better in terms of coping with the threat of deleterious mutation, however, there is a greater penalty to pay in terms of missing cells unavailable for somatic function.

4. Volvocalean green algae as a study case

4.1. The volvocalean green algal group

We use the volvocalean green algal group to test our model for the evolution of an early segregated germ-line. The volvocalean green algae comprise both unicellular (*Chlamydomonas*-like) algae as well as colonial forms in different stages of organizational and developmental complexity (Fig. 5). Interestingly, both multicellularity and germ/soma separation have evolved multiple times in this group. The different levels of organizational and developmental complexity are thought to be alternative stable states (Larson et al., 1992). Nevertheless, despite their multiple and independent acquisition of the multicellular state and germ/some separation, none of these multicellular lineages attained high levels of complexity, as did the green algal ancestors of land plants, the charophytes. We believe that understanding this limited spurt of diversification in complexity in this lineage will provide insight into how a transition to multicellularity may affect the evolvability of the new multicellular unit (Nedelcu and Michod, 2003). We suggest here that, although a germ-line acts to mediate conflict between

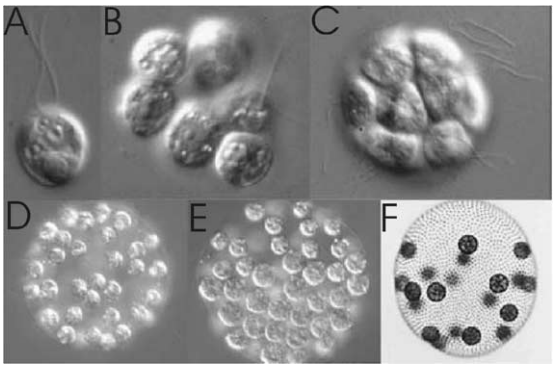

Fig. 5. The volvocine lineage. A subset of colonial volvocalean green algae that show a progressive increase in cell number, volume of extracellular matrix per cell, division of labor between somatic and reproductive cells, and proportion of vegetative cells. A: *Chlamydomonas reinhardtii*; B: *Gonium pectorale*; C: *Pandorina morum*; D: *Eudorina elegans*; E: *Pleodorina californica*; F: *V. 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.

the group and cell levels, and is thereby expected to contribute to the evolvability of the new multicellular group, the way in which the germ and somatic-lines are created may nevertheless interfere with the longer term evolvability of the lineage.

The basic morphological and developmental traits of the volvocalean green algae appear to result from the interaction of conflicting structural constraints (imposed by two very conserved anatomic traits) as well as two strong selective pressures. The first structural constraint is the so-called “flagellation constraint” (Koufopanou, 1994) which leads to a trade-off between reproduction and motility. This constraint has a different structural basis than the one invoked in the origin of metazoans (Margulis, 1981; Buss, 1987), although the end result is similar. In most green flagellates, during cell division the flagellar basal bodies remain attached to the plasma membrane and flagella, and can act as centrioles (which is not the case in animal cells); 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). Consequently, cell division and flagellar motility can take place simultaneously only for as long as flagella can beat without

having the basal bodies attached (i.e. up to five cell divisions). The second constraint comes from the unique way of cell division in volvocalean green algae, namely, the multiple fission type of division termed palintomy: cells do not double in size and then undergo binary fission; rather, cells grow about 2^n-fold in volume, and then rapidly undergo a synchronous series of n divisions (under the mother cell wall). 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).

The two selective pressures that are thought to have favored an increase in complexity in volvocalean algae are the advantages of a large size and the need for motility (Bell, 1985; Kirk, 1998). Larger size is thought to be advantageous by allowing colonies to escape predators, move faster, maintain better homoeostasis or better exploit eutrophic conditions, while motility allows the colonies better access to the euphotic/photosynthetic zone. Interestingly, as discussed next, given the ancestral constraints just mentioned, namely the flagellar constraint and the multiple fission type of cell division, it is difficult to achieve the two selective advantages simultaneously. Larger size via higher number of cells requires increased total time for cell division and, when coupled with the flagellation constraint, this means decreased motility.

4.2. Germ/soma separation in Volvox

4.2.1. Overview

As the volvocalean colonies increase in size and number of cells, the number of cell divisions as well as the size of the mature reproductive cell also increase. Due to the flagellation and palintomic constraints discussed above, 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 photosynthetic zone. For instance, in a *V. carteri* mutant with only 256 cells and no germ/soma separation (discussed later), the flagellar motility of the colony is negatively affected for as much 70% of the life cycle (i.e. 49–50 h in a 72-h life cycle); its decreased motility might be responsible for this mutant not being found in nature. In short, the need for the colony to increase in size detracts from the motility of the colony.

This negative impact of the flagellation and palintomy constraints was likely overcome by a division of labor (cellular differentiation) in the colony: some cells became specialized for motility, while others were assigned to reproductive functions. The proportion of cells that maintain motility and become sterile is directly correlated with the total number of cells in a colony: from none in *Chlamydomonas*, *Gonium*, and *Eudorina*, to up to one-half in *Pleodorina* and over 99% in *Volvox* (Larson et al., 1992). In the latter, the division of labor is complete: only the gonidia (the reproductive cells) undergo cleavage to form new colonies; the 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, 1982).

Volvox represents the most complex multicellular form in the volvocine lineage (Fig. 5). Germ/soma separation evolved at least twice, but possibly several times in *Volvox* (Kirk, 1999) and is realized differently among the at least 18 recognized species of *Volvox* (Desnitski, 1995). In some *Volvox* species, including *V. carteri*, the two types of cells are set apart by asymmetric divisions early in the embryonic development. Moreover, in *V. carteri* a difference in size, not a difference in cytoplasmic quality, determines which pathway of differentiation a cell will follow (Kirk et al., 1993). It should be mentioned that our discussion later is relevant only to this particular species of *Volvox*.

4.2.2. A comparative view

Many multicellular organisms have a germ-line that is segregated early in the development (for a list see for example Table 1.1 in Buss, 1987). Among these, the nematode worm *Caenorhabditis elegans* and the green alga *V. carteri* have been characterized in detail in terms of their development. Interestingly, although the number of somatic cells is small in both organisms (i.e. 959 cells versus 2000–4000, respectively), there are major differences between the two species with respect to the way in which germ-line is produced (Fig. 6) and the number of cell types (more than 20 in the former and only 2 in the latter). What might be the evolutionary reasons for such differences? Later, we describe features of development in the two lineages, contrast them, and point out the peculiarities of germ/soma separation in *V. carteri*.

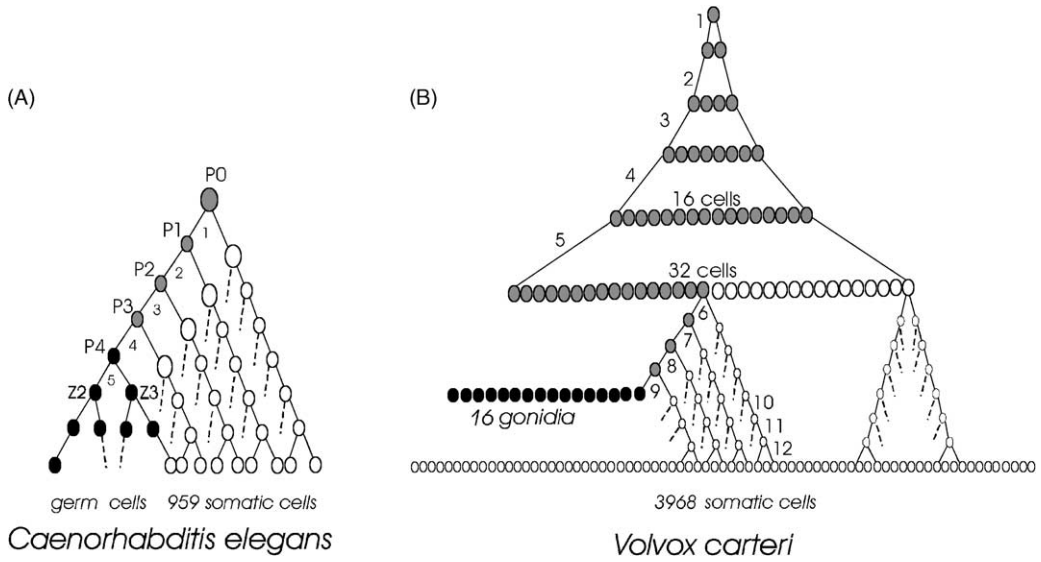

Fig. 6. Two examples of early germ-line segregation. (A) Germ-line segregation in the nematode worm *C. elegans*; gray, black, and white ellipses indicate germ-line blastomeres, primordial germ cells, and somatic blastomeres, respectively. P0 denotes the zygote; P1, P2, P3, are the germ-line blastomeres; P4 is the germ-line founder cell; Z2 and Z3 are primordial germ cells. Numerals indicate successive cell divisions. (B) Germ-line segregation in the green alga *V. carteri*; gray and white large ellipses denote totipotent and somatic blastomeres, respectively; gray, black, and white small ellipses indicate germ-line blastomeres, gonidia, and somatic initials/cells, respectively. Details for the division pathways are shown only for one germ-line founder cell and one somatic blastomere. Numerals mark successive cell divisions.

The first four cell divisions during the *C. elegans* embryonic development are asymmetric (e.g. Seydoux and Schedl, 2001). The zygote, P0, as well as the smaller cells resulting from the first three asymmetric divisions (i.e. P1–P3 in Fig. 6A) act as stem cells: with every asymmetric cell division they produce another totipotent stem cell (they renew themselves) and a somatic blastomere. These stem cells, or germ-line blastomeres, are the precursors of the germ-line. The last of these cells, P4, represents the founder cell (or progenitor) of the germ-line. Ultimately, the P4 cell divides symmetrically to produce two primordial germ cells (PGCs), Z2 and Z3, that migrate to the gonad and arrest mitosis until the larva hatches; later on, Z2 and Z3 proliferate and undergo meiosis to produce the germ cells. What specifies the fate of the cells following the four asymmetric divisions is a set of germ-line granules, or P granules, which are present in the zygote and are selectively distributed only to the germ-line precursors and ultimately to the two PGCs (e.g. Seydoux and Schedl, 2001). The larger cells resulting from the first four asymmetric divisions act as somatic blastomeres or founder cells, and proliferate to form various cell types, such as neurons, epithelial, muscle, gland cells, etc.; some of the divisions responsible for the 959 somatic cells in the adult take place after the larvae hatch.

In contrast to *C. elegans*, the first divisions in *V. carteri* are symmetrical (Fig. 6B). Up to the 16-cell stage, all the blastomeres are totipotent (if removed they can create a new colony); after the fifth division, the 16 cells situated at the anterior pole of the embryo will take a distinct path relative to the 16 cells at the posterior pole. The first difference is that the sixth cell division is asymmetric in the 16 anterior cells but symmetric in the other 16 cells. What determines which cells will undergo an asymmetric division is not well understood; physical cues (i.e. physical constraints) associated with the number of cytoplasmic bridges and the position of cells in the embryo are thought to be involved in this process (Kirk, 1994). By having the first blastomeres functionally equivalent (i.e. totipotent) and by relying on spatial cues for initiating the pathway leading to the differentiation of the germ-line, *V. carteri* differs from *C. elegans* but resembles mammals (e.g. Anderson et al., 2001).

However, the further steps in the segregation of the germ-line are rather unique in *V. carteri*.

The 16 cells predetermined to produce the germ-line are totipotent, act as stem cells and undergo a series of three–four successive asymmetric cell divisions, in a manner similar to that of the *C. elegans* germ-line precursors (germ-line blastomeres P0–P3) (Fig. 6). Nevertheless, the similarities stop here. In contrast to *C. elegans*, in *V. carteri*, the larger cell resulting from the asymmetric division is the one to act as a germ-line blastomere. Furthermore, the asymmetric division is not accompanied by a differential segregation of a specific cytoplasmic component; what triggers the somatic fate of the smaller cells in *V. carteri* is not the lack of distinct cytoplasmic components, as it is in *C. elegans*, *Drosophila*, and *Xenopus* (e.g. Kloc et al., 2001; Mahowald, 2001; Seydoux and Schedl, 2001), but rather strictly a difference in cell size (Kirk et al., 1993). In addition, the large cells that result from the ninth division (and which are analogous to the *C. elegans*'s progenitor of the germ-line, P4) do not divide to produce mitotically active primordial germ cells, PGCs. Rather, these cells arrest mitosis (while the somatic blastomeres continue to divide for another two–three cycles) and differentiate without further divisions into single non-flagellated germ cells, gonidia. Nevertheless, although each germ-line founder cell produces only one germ cell (gonidium), it is interesting that there are up to 16 such cells in each spheroid, as if there were 16 independent germ-lines each producing a single germ cell. On the other hand, the smaller cells produced though asymmetric cell divisions in the anterior pole as well as the cells produced by the 16 posterior blastomeres stop dividing while still in the embryo, and all differentiate into the same type of cell, i.e. flagellated somatic cells.

5. Germ/soma separation and evolvability during the transition to multicellularity

We have discussed elsewhere how the transition from unicellular to multicellular life requires the decoupling of basic life-properties at the lower level and their re-coupling and re-organization in new ways at the higher level (Nedelcu and Michod, 2003). Moreover, we argued that some of the differences among the extant species, including differences in evolvability, can be explained by the way in which the decoupling and re-coupling of these properties has been achieved during the transition to multicellularity.

Two of the complex life-traits that become reorganized during the unicellular–multicellular transition are immortality and totipotency. By "immortality" we mean the continued capacity for cell division and by "totipotency" we mean the capacity of a cell to create a new organism. In unicellular organisms, these traits are necessarily coupled at the cell level in the sense that they are both fully expressed in all cells. In multicellular organisms, however, the two traits are re-organized within and between cell lineages. In species with defined germ and somatic-lines, the two traits are fully expressed only in the germ-line, whereas somatic cells obviously do not express totipotency and express continued replicative ability, that is immortality, to varying degrees. In *V. carteri*, immortality and totipotency are fully restricted to the germ-line and somatic-lineages have no mitotic or replicative potential (Nedelcu and Michod, 2003). The un-coupling of immortality and totipotency proved not possible in *V. carteri:* these traits are express either together and fully (i.e. in the gonidia) or not at all (i.e. in the somatic cells). Immortality and totipotency are thus still tightly linked in *V. carteri*, as they are in their unicellular ancestors. In support of this view is the fact that "cancer-like" mutant somatic cells, in which immortality but not totipotency is re-gained, are missing in *V. carteri*. There are, however mutant forms of *V. carteri* (discussed later) in which somatic cells re-gain both immortality and totipotency, but in neither of these mutants are the two traits expressed partially or differentially (e.g. limited mitotic capacity or multipotency).

To ensure the emergence of individuality at the higher level and the reproduction of the multicellular individual (i.e. the heritability of the group-level traits), immortality and cell division have to be de-coupled from the reproduction of the lower-level cells (previously unicellular individuals) and be co-opted for the reproduction of the group (the higher-level multicellular unit). Furthermore, in lineages with a separation between germ and soma, in somatic cell lineages, cell division is not associated with reproduction of the individual but rather became co-opted for growth of the multicellular individual (Nedelcu and Michod, 2003).

V. carteri avoided the risks and potential conflicts of cell division in somatic cells by blocking it altogether and in this way achieved individuality at the cell-group level. In addition cell division was not co-opted for the post-embryonic growth of the multicellular individual. We believe that this solution to the mediation of conflicts at the lower level affected the long-term evolutionary adaptability of the lineage. Later, we investigate the possible reasons for undertaking this peculiar pathway. Our fundamental point is that, although conflict mediation is pivotal to the emergence of individuality at the higher-level, the way in which the mediation is achieved during the transition in individuality can interfere with the long-term evolvability of the lineage.

6. The evolution of a germ-line in *Volvox carteri*

6.1. The question

In many lineages, including *C. elegans* for instance, the germ cells are the descendants of a single germ cell founder (e.g. P4 in *C. elegans*; Fig. 6A). In contrast, as we discussed earlier, in *V. carteri*, the 16 germ cells are formed from 16 independent germ-line blastomeres that stop dividing and differentiate directly into gonidia (Fig. 6B). Theoretically, the same 16 gonidia could be produced from a single founder cell (as is the case in *C. elegans*) by dividing a total of four times ($1 \times 2^4 = 16$); this raises the question as to why in *V. carteri* the germ cells differentiate from 16 independent germ-line founder cells instead of only one germ founder cell.

To address this issue, we envisioned for *V. carteri* a developmental pattern similar to that of *C. elegans*, and asked what the consequences would be. Surprisingly, such a pathway would be identical to the current one in terms of the numbers of gonidia and somatic cells (Fig. 7). We envisioned that after the fifth cell division (which is the time where the 16 germ-line blastomeres are determined in *V. carteri*), only one cell is sequestered, and would divide symmetrically a total of four times to produce 16 gonidia; the other 31 cells undergo a path identical to that of the 16 posterior cells in *V. carteri*, that is they divide symmetrically seven times to produce somatic cells (Fig. 7). Interestingly, by having only one cell acting as a germ-line founder and 31 (instead of 16) cells acting as somatic blastomeres, the same total of 3968 somatic cells (i.e. $31 \times 2^7 = 3968$) and 16 gonidia (i.e. $1 \times 2^4 = 16$) would be produced. Furthermore, under this scenario, asymmetric divisions are not required (see discussion later). Then, why was the former alternative favored over the latter, especially when it requires the evolution

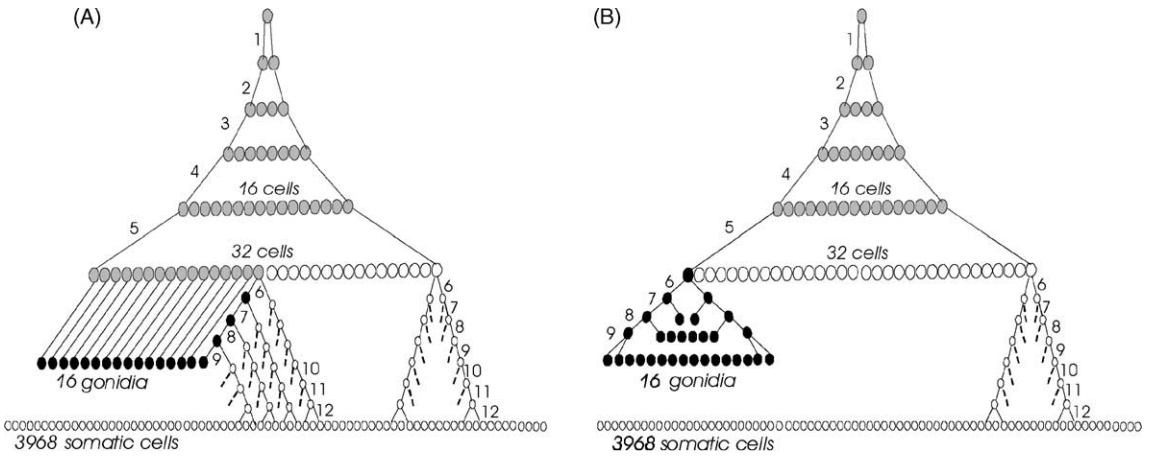

Fig. 7. Two alternative scenarios for germ-line segregation in *V. carteri*. (A). The alternative that has been selected for in *V. carteri*: germ cells differentiate from 16 independent germ-line founder cells. (B). Our theoretical alternative: germ-line cells derive from a single germ-line founder cell. Symbols are as in Fig. 6.

of a new trait, namely, the asymmetric type of cell division?

6.2. Applying the model

Can the model on the evolution of an early segregated germ-line presented earlier help address this question? We use our model to ask under which conditions would one or the other alternative diagrammed in Fig. 7 be selected for, so as to understand the "choice" that *V. carteri* has made.

6.2.1. Assumptions

In trying to understand the various selective factors, we assume that 12 cell divisions is the highest number of cell divisions possible under the multiple-fission palintomy type of cell division. In reality, this is true for *V. carteri*. As discussed earlier, with the multiple-fission palintomy, to reproduce a colony with 2^n cells, a single reproductive cell must first grow 2^n before it divides n times. Clearly, a single cell cannot grow indefinitely in size (especially if it is a spherical shape) without affecting its metabolic abilities (due to changes of the surface/volume ratio). It appears that a 500–1000-fold increase in size is the most a gonidia can stand without affecting its metabolism, and 2^{12} cells is the highest number of viable cells that can result from such a gonidia. We do not have a detailed argument for this assumption, however there are no palintomic *Volvox* species in which n is higher than 12. Nevertheless, in *Volvox* species that have "escaped" the palintomic constraint, n can be higher than 12. As a consequence, gonidia do not have to grow up to 2^n and then divide n times, rather they only double in size, divide, grow between divisions, divide again and so on. Interestingly, in the non-palintomic species, gonidia differentiate late in development, suggesting a correlation between palintomy and early-segregation of germ (i.e., the fast cell division cycles associated with palintomy may cause higher mutation rates).

6.2.2. Cost of the germ-line

We interpret the cost of the germ-line in terms of the missing somatic cells that are unavailable for vegetative functions (as expressed in Eq. (3)). In *V. carteri*, such a cost is likely very important, due to the selective pressures mentioned previously, namely, the need for daily vertical migrations in the water column and a large body-size. The presence of a non-flagellated germ-line affects the fitness of the group by interfering with the mobility of the group through decreasing the number of cells that participate in motility (i.e. by affecting the ratio of flagellated to non-flagellated cells). In addition, an early segregated and arrested germ-line decreases the overall number of cells in the group and thus the body-size is affected.

The time of sequestration as well as the number of cell divisions following the sequestration event influence the cost of the germ-line both in terms of the total number of cells missing in the group as well as the missing soma, that is missing flagellated cells. We illustrate the trade-offs involved with three examples. First, if only one germ-line founder cell is sequestered after the first embryonic cell division (as in our earlier model) and continues to divide for the same 12 times as the somatic-lineages, the total number of cells would not be affected (i.e. 4096), but the flagellated to non-flagellated cell ratio would be 1:1 and thus the motility of the group would be greatly affected. In this case, the cost of the germ-line is only in terms of the number of flagellated cells missing (and not overall colony size), but the cost is very high (i.e. 2048 missing motile cells). In addition, the germ cells would likely accumulate the same number of mutations as the somatic cells assuming mutation is dependent on the number of DNA replications. Second, if the founder cell segregated after the first cell division and underwent only four cell divisions to produce 16 gonidia (and then arrest mitosis while the somatic-lineages continue dividing for a total of 12 times) the group would be composed of only 2048 somatic cells and 16 gonidia. In this case, the ratio increases in the favor of the motile cells (2048:16), but the colony size is affected (2064 versus 4096 cells). The cost of having a germ-line segregating very early and arresting mitosis after four cell divisions is very high in this example, both in terms of total number of cells (i.e. 2032 missing cells) as well flagellated cells (i.e. 2048 cells). However, germ cells would have undergone a fewer number of cell divisions than the somatic cells and would have fewer mutations as a result. Third, at the other extreme, if the founder cell is put aside only after the eighth division and undergoes four additional cell divisions to produce the 16 gonidia, the cost would only be in terms of the flagellated cells, which

is very low (i.e. 16 cells). Nevertheless, the germ cells would be dividing for the same number of times as the somatic cells.

A compromise may be reached between group size and percentage of flagellated cells by having the germ-line segregate at an intermediate stage during development and arresting mitosis earlier than in the somatic-lineages, such that both the cost in terms of missing cells and the mutation levels are lower. If only one founder-germ cell segregates after the fifth division (Fig. 7B) and undergoes a number of additional four divisions to produce the 16 gonidia, the cost of germ-line in terms of total number of cells is 112 cells ($4096 - 3984 = 112$) and in terms of flagellated somatic cells is 128 cells ($4096 - 3968 = 128$). In addition, there is a 25% decrease in the number of cell divisions in the germ cells relative to somatic cells (9 versus 12), which might result in a lower mutation level in the germ-line. However, as discussed later, the same low cost can be achieved through either the scenario just discussed or the pathway that *V. carteri* is actually following (Fig. 7A); why the latter was selected for is discussed next.

6.2.3. Parameters and values

We used two sets of values for the model's parameters: first, the values used by *V. carteri*, and second, the values that correspond to the theoretical alternative we envisioned in Fig. 7B. In both cases, the somatic cells divide for up to 12 times, thus $t = 12$ in our model. Likewise, the cost of the germ-line is the same in both cases following that assumed in Eq. (3) earlier; if all cells remained undifferentiated and replicated 12 times, there would be 4096 cells in the group, so there are 128 (i.e. 4096 – 3968) cells missing in the soma. We assume asexual reproduction ($r = 0$), a significant benefit of cooperation (coming from motility) $\beta = 10$, and that the mutation rates per cell division are equal in the germ and soma, $\mu = \mu_G$. In addition, for each set of conditions, we used two values for the mutation rate, $\mu = 0.003$ and an order of magnitude higher $\mu = 0.03$ (and, as already mentioned, the number of missing somatic cells is the same in both cases). The difference between the two situations concerns the sampling of mutations and the resulting opportunities for conflict between levels of selection.

The parameters that differ in value between the two cases are the time of sequestration (θ), the number of germ-line cell divisions after sequestration (t_G), and the number of cells sampled (i.e. germ-line founders, ν). In the first case (Fig. 7A), there are 16 germ-line founder cells ($\nu = 16$) that segregate from the somatic-lineage after the ninth cell division ($\theta = 9$) and arrest mitosis at that very point ($t_G = 0$). In contrast, in the second case (Fig. 7B), there is only one germ-line founder cell ($\nu = 1$) that segregates earlier ($\theta = 5$) and divides for an additional four times ($t_G = 4$). In both cases, the total number of cell divisions that gonidia undergo is nine, while the additional number of cells divisions in the somatic-lineage is three ($\delta = 3$).

6.2.4. Predictions

Fig. 8 depicts the results of our model with both sets of values (panels A and C versus panels B and D) and under two mutation rates (panels A and B versus panels C and D). Interestingly, the model predicts that a germ-line is easier to evolve (the germ-line evolves for a greater range of parameter values) under the second set of parameter values, namely, those corresponding to a germ-line descending from a single germ-cell founder, the route not taken in *V. carteri*. In both cases, the model predicts that the germ-line evolves easier when mutation is a threat, either because mutations are selfish ($b > 1$) or frequent (a high mutation rate). When mutations are frequent, cooperation is harder to maintain, and so the within-group advantage for the mutant cells (represented by parameter b) has to be lower. Under a lower mutation rate, a germ-line can evolve only when the within-group advantage of mutant cells is rather high, approaching 1.2 in the first case, or 1.1 in the second case. Also, in the first case (Fig. 8 panel C), a germ-line evolves only if the number of cell divisions after segregation is very small, approximately t_G must be less than 2. These conclusions are mirrored in Fig. 4. Panel C of Fig. 4 shows that t_G (fecundity) can be higher with $\nu = 1$ and $\theta = 5$ than with $\nu = 16$ and $\theta = 9$, and this explains the results of Fig. 8. It is always good to decrease ν (the initial number of germ cells), and it is good to decrease θ up to a certain point, after which there are not enough somatic cells (this is why we see a maximum on Fig. 4C). So we can not say a priori if $\nu = 1$ and $\theta = 5$ is better than $\nu = 16$ and $\theta = 9$

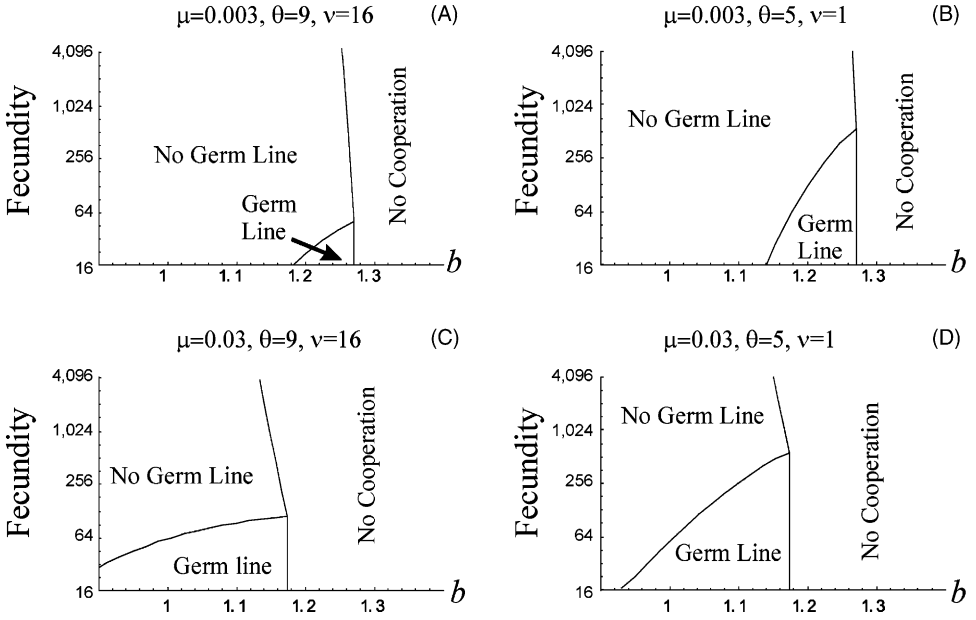

Fig. 8. Evolution of germ-line segregation in *V. carteri*. The main contrast in the figure is between a germ-line developed from 16 founder cells sequestered at cell division 9 (panels (A) and (C)) and a germ-line from a single founder cell sequestered at cell division 5 (panels (B) and (D)). The ordinate is t_G (as it is in Figs. 3 and 4) except expressed in terms of fecundity, that is the number of resulting gonidial germ cells. For example, if a single cell is sequestered ($\nu = 1$) to divide five times ($t_G = 5$), fecundity is 16 gonidia. Likewise, if 16 cells are sequestered ($\nu = 16$) to divide no times ($t_G = 0$), fecundity is 16 gonidia. The mutation rate is an order of magnitude higher in the bottom panels than in the top panels. In the region labeled "no cooperation" group living cannot be maintained. In the two other regions, cooperative groups are maintained with or without a germ-line. All panels assume fitness Eq. (3) (cost of the germ-line results from missing somatic cells), equal mutation rate in germ and soma ($\mu = \mu_G$), no sex ($r = 0$), benefit of cooperation $\beta = 10$, number of cell divisions in soma $t = 12$. Recall t_G is the number of cell divisions in the germ-line after sequestration and b is the within-group advantage of mutants. Noting $\delta = t - t_G$, compare Figs. 6–1 (Michod, 1999), Fig. 1 (Michod and Roze, 1997), Fig. 6 (Michod and Roze, 1999). Methods used to construct the graphs are given in the Appendix A.

(because of the non linear effect of θ), but Figs. 4 and 8 show that it should be better. However, the developmental biology of *Volvox carteri* does not agree with this prediction of the model.

In summary, the model predicts that in *V. carteri* a germ-line is easier to evolve if mutant cells are selfish (but not so selfish that cooperation cannot be maintained), or if the mutation rate is rather high (Fig. 8). Are any of these conditions met in *V. carteri*? Moreover, the model suggests that the scenario based on a single germ-cell founder (Fig. 7B) should be easier to evolve than the one that has actually evolved in *V. carteri* (Fig. 7A). Then, why was the germ-line in *V. carteri* not formed under the conditions (i.e. a single germ-cell founder) that the model predicts are most conducive for the evolution of a germ-line? Below we discuss these issues.

7. Discussion

7.1. Selfish mutants

According to the model (Fig. 8), a germ-line is predicted to evolve only when the advantage of within-group mutations is rather high. This is consistent with the fact in *V. carteri* many mutations that affect the somatic cells are selfish. In the somatic regenerator mutants, or Reg mutants, the somatic cells start out as small flagellated cells (wild type-like) 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., 1999b). 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 (by repressing chloroplast biogenesis in these cells (Meissner et al., 1999a)) enough to trigger cell division. In another class of mutants, the Gls/Reg mutants (Huskey and Griffin, 1979), all the cells (though 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 resemble volvocacean species with no germ/soma separation, such as *Eudorin*a. The *Gls* mutation has been mapped to a gene, *gls*A, that encodes a protein required for the asymmetric divisions responsible for the segregation of germ-line and somatic blastomeres (Fig. 5A) (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 such that the growth of somatic cells and thus their differentiation into reproductive cells is not suppressed.

The expression of *RegA* and *Gls*/*Reg* mutations has a profound effect on the higher-level unit, that is the colony. While the somatic cells re-differentiate into gonidia, the spheroid is unable to maintain its motility (and thus its position in the euphotic zone) for more than the duration of the first five cell divisions; however, the total number of cell divisions required to reach the final number of cells in the embryo is seven or eight, and eleven or twelve in the Gls/Reg and Reg mutants, respectively. In conditions where motility and access to light are strong selective pressures, the higher-level is negatively affected by the occurrence of these mutant cells at the lower-level, which are thus acting as “selfish” mutant cells. To argue for the negative effects at the higher-level of these types of selfish mutations in the environments where wild-type forms of *Volvox* are usually present is the fact that neither of the mutant forms are found as established populations in nature, although the Reg mutants occur spontaneously at a rather high rate (Kurn et al., 1978). Interestingly, however, when access to light and the need for motility are not representing selective pressures (i.e. in lab settings or possibly shallow waters), the fitness of these mutant forms proves higher than the fitness of the wild-type (Koufopanou and Bell (1991) and our unpublished data).

7.2. Mutation rates

In our model, we used a value for the genome wide mutation rate reported for unicellular organisms such as yeast, bacteria, viruses (Drake, 1991), yet the model studies only a single locus assumed to represent the genome wide functions involved in cellular functions. Our use of a genome wide rate in a single locus model is clearly a leap, but it gives an idea of what we hope the model represents. We have considered more realistic mutation models elsewhere (Michod and Roze, 2000; Roze and Michod, 2001). Concerning the higher mutation rate assumed in panels C and D of Fig. 8, it is worth noting that volvocalean green algae seem to feature levels of nucleotide substitution (as suggested by the differences in branch length observed in phylogenetic trees based on nuclear rRNA sequences (e.g. Friedl, 1997; Nakayama et al., 1998) that are higher than those in other green algae as well as in their close relatives, the land plants, which, incidentally, do not have an early-defined germ-line.

7.3. Sixteen versus one germ-line founder

Although our model predicts that in both cases a germ-line is more likely to evolve if the mutation rate and the threat of selfish mutations are higher, these conditions are more relaxed under the scenario in which gonidia descend from a single germ-line cell founder (Fig. 8B), instead of 16 different founder cells as is the case in *V. carteri*. Why is this scenario not the one that was selected for in *V. carteri*? Interestingly, although the end result, as far as the composition (i.e. number and type of cells) of the colony, is the same under both scenarios, two aspects are different and not represented in the model.

One aspect not represented in the model is asymmetric cell division. The second scenario (Fig. 7B) does not use or require asymmetric divisions. However, the scenario that has been selected for in *V. carteri* involves asymmetric divisions; so, why did asymmetric division evolve in *V. carteri*? Asymmetric division

is considered a derived trait (Desnitski, 1992) in the volvocalean green algal group. Asymmetric divisions do not occur in the less complex *Pleodorina* as well as many species of *Volvox*; in these cases, all gonidia are developed from cells that were initially indistinguishable from somatic cells, but then undergo enlargement and differentiation. In *V. carteri*, asymmetric divisions and the segregation of the germ-line are not associated with the differential segregation of cytoplasmic components (Kirk et al., 1993). Rather, it has been shown that the cells that remain above a threshold (i.e. 8 μm) differentiate into germ-cells, whereas the cells that fall below the threshold will take the somatic path (Kirk et al., 1993), regardless of the type of cell division. To argue for this is also the fact that the formation of the 2048 somatic cells produced by the 16 posterior blastomeres does not involve any asymmetric divisions.

Interestingly, in the Gls/Reg mutant, all of the cell divisions are symmetric; the cells follow the ancestral *Chlamydomonas* pathway, acting first as vegetative cells and then re-differentiating into reproductive cells. At the end of the seventh or eighth cell division (which marks the end of the embryonic cleavage in this mutant form that only has 128 or 256 cells), all the cells are about 4 μm in size. At the equivalent time in development, the somatic initials in the wild-type form are of the same size. However, due to the lesion present in the *regA* gene, the Gls/Reg mutant cells are able to grow post-embryonically and re-differentiate into reproductive cells, whereas the wild-type somatic cells, in which the *regA* gene is active, are destined to remain small and terminally differentiated.

Thus, it is likely that under the scenario favored by our model (Fig. 7B), subsequent to the seventh or eighth symmetric cell division, the cells would fall below the threshold size and would terminally differentiate into somatic cells; consequently, there would be no reproductive cells to ensure the formation of progeny. This problem could be resolved by having the germ-line founder cells undergo fewer cell divisions, and by having more than one founder cells; for instance, four instead of one germ-line founders that would undergo a number of only two additional divisions would produce the same total number of 16 gonidia (4×2^2) and the critical cell size would not be reached because the total number of cell divisions would not exceed seven ($5 + 2 = 7$). However, the embryo would be short of 384 somatic cells ($3 \times 2^7 = 384$). Under the selective pressure to achieve a large size and high motility, such a decrease in the total number of cells in the adult might be disfavored. Then, how can the number of cells be increased while still producing 16 germ cells? Asymmetric divisions are able to do that by keeping the size of one (i.e. the germ cell blastomeres) of the two daughter cells above the threshold, while producing additional somatic blastomeres that continue to proliferate below the threshold, and thus increase the total number of somatic cells (Fig. 7A). Therefore, the path that *V. carteri* undertook can be explained as a consequence of the need to produce the maximum number of germ cells and reach the largest number of somatic cells, under the constraint of palintomy (i.e. no cell growth between cell divisions) and the particular mechanism underlying somatic cell differentiation in this species (i.e. the expression of *regA* when cell size falls below a threshold).

The other aspect that distinguishes the two alternatives for segregating a germ-line in *V. carteri* is concerned with the degree of relatedness among gonidia; the gonidia are less related to each other under the first scenario when compared to the second. It is possible that by having the 16 germ cells deriving from 16 independently segregated germ-line founder cells, an increase in genetic variance among the progeny is achieved. Because this species is reproducing mainly asexually/clonally, such a pattern of producing the germ cells might contribute to achieving genetic variation in populations. On the other hand, it has been suggested that one of the advantages of having an early segregating germ-line comes from the reduction in the mutation level either by lowering the number of cell divisions (Buss, 1987) or by lowering the mutation rate per cell division (Michod, 1996, 1999; Michod and Roze, 2000). It is interesting to note that the way that the germ cells are produced in *V. carteri* ensures that the deleterious mutations that might occur during the last four cell divisions (i.e. the sixth, seventh, eighth, and ninth) will be restricted to only one of the 16 germ cells (Fig. 7A). In contrast, in the conventional way of proliferating germ cells from only one germ-line founder, deleterious mutations that would occur during the sixth division would be transmitted to half or a quarter of the progeny (depending on whether the mutation affects both or only one of the DNA strands, respectively). In this light, the special way that the germ-line is formed in *V. carteri* may be considered

as a means to allow the accumulation of non-lethal variation, which is one of the premises for achieving evolvability.

8. Germ-line, conflict mediation and evolvability

Although an early-segregated germ-line and a soma have evolved in *V. carteri*, the soma was achieved in a rather peculiar way. *Volvox* was not able to re-organize immortality and totipotency among its cell lineages; instead, in *Volvox*, both of these traits are entirely suppressed in the somatic cells. Moreover, the suppression of these traits was achieved by acting on a single process, namely cell division. Furthermore, the way in which *Volvox* suppressed cell division was not by acting directly on the mitotic potential of the cells but rather indirectly by acting on a trait that was still very linked to it, that is the growth of the cell (Nedelcu and Michod, 2003). By suppressing cell growth in somatic cells, cell division is repressed and the potential for re-gaining immortality and totipotency (i.e. and for gaining access to the germ-line) is "under control." We suggest that it is this type of conflict mediation affected the potential for further evolution in this lineage.

A direct implication is that "soma" in *Volvox* differs from the soma of other multicellular organisms. Because somatic cells do not divide, the post-embryonic growth and/or regeneration of the individual are not possible; in addition, because the somatic cells undergo senescence and cell death at the age of 5 days (Pommerville and Kochert, 1981, 1982), the life span of the higher-level individual is limited to the life span of the lower-level somatic cell. 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). Without a mitotically active multipotent stem cell lineage or secondary somatic differentiation there is less potential for cell differentiation and further increases in complexity. However, somatic growth and differentiation are important for the evolvability of a multicellular lineage. Without them, *Volvox* did not and will likely not attain higher-levels of complexity.

In conclusion, we have tried to understand how developmental processes are shaped during evolutionary transitions to increase the evolvability of the new higher-level unit. In our formulation, evolvability of the new unit of selection (multicellular organism) depends on enhanced cooperative interactions among lower-level units (cells). Conflict mediation, the process by which cooperative interactions are enhanced by reducing the temptation to defect, is instrumental in creating a new evolutionary individual. Conflict mediation increases the cooperativity of the group and heritability of fitness at the new level. Upon such processes does the continued evolvability of the new unit evolutionary unit depend. Nevertheless, conflict mediation, like other selective processes, can be short-sited and in the case of *V. carteri* appears to have interfered with the long-term evolvability of the lineage.

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Appendix A

The 2-locus 2-allele haploid dynamical system underlying this study has been introduced elsewhere (Michod, 1997, 1999; Michod and Roze, 1997). In the present study, we assume no recombination, $r = 0$. We consider two loci, the first locus controls cell behavior (alleles C for cooperate and D for defect) and the second modifier locus controls some aspect of development or organization of the system (alleles m for the ancestral state and M for the modified state). Here the modifier allele M creates a sequestered germ-line. There are four genotypes CM, Cm, DM, and Dm, referred to as genotype 1–4, respectively, in the subscript notation later.

In an organism with a germ-line (cell groups coming from a CM zygote) there is a distribution of C and D cells at the time θ when the germ-line differentiates. We define $k_{11,\theta}$ the number of CM cells at the time θ, $k_{31,\theta}$ the number of DM cells and $k_{1,\theta}$ the total number of cells ($k_{1,\theta} = k_{11,\theta}, k_{31,\theta}$) coming from a CM zygote. N_{C} is the number of C cells in the initial sample of ν cells which will give the germ-line and is given by a hypergeometric distribution

$N_C = H(k_{1,\theta}, \nu, (k_{11,\theta}/k_{1,\theta}))$. So we have for all i between 1 and ν,

$$P_i = \Pr(N_C = i) = \frac{\binom{k_{11,\theta}}{i}\binom{k_{31,\theta}}{\nu - i}}{\binom{k_{1,\theta}}{\nu}}.$$

From our previous work (Michod, 1996; Michod and Roze, 1997), we know the values of $k_{11,\theta}$, $k_{31,\theta}$, $k_{1,\theta}$ at the time of the germ-line sample.

$$k_{11,\theta} = 2^{c\theta}(1-\mu)^{c\theta},$$

$$k_{31,\theta} = \frac{\mu 2^{bc\theta} - 2^{c\theta}(1-\mu)^{c\theta}\mu}{-1 + 2^{b-1} + \mu},$$

and $k_{1,\theta} = k_{11,\theta} + k_{31,\theta}$. Here μ is the mutation rate per cell division from alleles C to D, c is the replication rate of D cells (often assumed unity for simplicity and without loss of generality) and cb is the replication rate of mutant D cells. As we assume an infinite population, we assume that P_i is the frequency of CM groups that will give i C cells.

The ν cells in the germ-line sample go on and divide for a time t_G to give the germ-line, and the mutation rate affecting the C allele is μ_G (a different rate, perhaps, if the germ-line precursor cells are treated differently). We call $K_{11,i}$, $K_{31,i}$ and $K_{1,i}$, the number of C cells, the number of D cells and the total number of cells in the germ-line of a CM individual whose sample had i C cells. We have

$$K_{11,i} = i \times 2^{ct_G}(1-\mu_G)^{ct_G},$$

$$K_{31,i} = i\frac{\mu_G 2^{bct_G} - 2^{ct_G}(1-\mu_G)^{ct_G}\mu_G}{-1 + 2^{b-1} + \mu_G} + (\nu - i) \times 2^{bct_G},$$

and $k_{1,i} = k_{11,i} + k_{31,i}$.

We call $k_{11,i}$, $k_{31,i}$, and $k_{1,i}$, the number of C cells, the number of D cells and the total number of cells in the soma of a CM individual whose sample for the germ-line had i C cells. We then have

$$k_{11,i} = 2^{ct}(1-\mu)^{ct} - i \times 2^{c(t-\theta)}(1-\mu)^{c(t-\theta)},$$

$$k_{31,i} = \frac{\mu 2^{bct} - 2^{ct}(1-\mu)^{ct}\mu}{-1 + 2^{b-1} + \mu} - i\frac{\mu 2^{bc(t-\theta)} - 2^{c(t-\theta)}(1-\mu)^{c(t-\theta)}\mu}{-1 + 2^{b-1} + \mu} - (\nu - i)2^{bc(t-\theta)},$$

and $k_{1,i} = k_{11,i} + k_{31,i}$.

The expression of fitness for a group initiated by a CM cell whose germ sample had i C cells is then

$$W_{1,i} = A\left(1 + \beta\frac{k_{11,i}}{k_{1,i}}\right),$$

with $A = 2^{ct}(1-\mu)^{ct} + ((\mu 2^{bct} - 2^{ct}(1-\mu)^{ct}\mu)/(-1 + 2^{b-1} + \mu)) - K_{1,i}$, if the cost of the germ-line is in terms of the new germ cells (Fig. 3), and $A = k_{1,i}$, if the cost is in terms of number of missing somatic cells (Fig. 4). Note that $k_{1,i}$, is the number of somatic cells after the germ-line has been sequestered and takes into account the cells that are missing.

To date, we have only studied the case of asexual reproduction, in which case the two-locus modifier model has four equilibria (given and discussed in Table 6–2 of Michod, 1999). The first equilibrium and second equilibria do not interest us here as they correspond to no cooperation (C allele in frequency zero) with the modifier allele at frequency unity or zero. The third and fourth equilibria correspond to a population polymorphic at the cooperate/defect locus with the modifier M allele in frequency zero, or unity, respectively. We are interested in the case when the germ-line modifier allele M increases in frequency and the system undergoes a transition from equilibrium three to equilibrium four. This transition corresponds to a transition in individuality, because the group of cells are no longer indivisible, some cells the somatic cells, can no longer produce the group. Instability of equilibrium three is determined by the following eigenvalue condition

$$\lambda = \frac{(k_{22}/k_2)W_2}{\sum_{i=0}^{\nu} P_i(K_{11,i}/K_{1,i})W_{1,i}} < 1.$$

If $\lambda < 1$, when the M allele appears in the population, its frequency goes to fixation, and the system goes from equilibrium three to equilibrium four. It is this condition that leads to the results in Figs. 3, 4 and 8.

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