Dasycladales Morphogenesis: The Pattern Formation Viewpoint

By

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B.Sc. (Biology) Université de Sherbrooke, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER IN SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF BOTANY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1996

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Date April 22nd, 96

DE-6 (2/88)

Abstract

The Dasycladalian algae produce diverse whorled structures, among which the best-known are the reproductive whorl (cap) and the vegetative whorls (hair whorls) of *Acetabularia acetabulum*. The origin of these structures is addressed in terms of three pattern forming mechanisms proposed to explain whorl formation. The mechanisms involve either: mechanical buckling of the cell wall, reaction-diffusion of morphogens along the cell membrane, or Ca^{2+}-cytoskeleton mechano-chemical interactions in the cytosol. They are described and their idiosyncrasies underlined to provide a ground to test them experimentally. It is also suggested that the closely regulated spacing between the elements of a whorl is a key component of such a test. A detailed staging of whorl formation in the genus *Acetabularia* shows that the elements constituting the whorl can be traced back to their initiation as localized wall lysis. Stagings of the genera *Polyphysa, Batophora, Halicoryne,* and *Neomeris* are also provided. The succession of wall thickening and wall lysis as well as the spacing observed are to some extent incompatible with the idea of wall buckling. The stagings show also the homology between the reproductive and vegetative whorls. Of the different homological systems proposed, one is singled out based on a re-interpretation of the gametophore as a *sui generis* organ instead of its more common interpretation as a modified hair. Based on this evidence and that provided by the fossil record, it is shown that a reduction of the spacing within a whorl, the addition of one morphogenetic event for the gametophore and a redistribution of growth are sufficient to explain the major differences between the vegetative and reproductive whorls of several genera. More attention is given to the seemingly exceptional case of *Halicoryne*. A study of membrane-bound and free Ca^{2+} distribution during morphogenesis reveals that Ca^{2+} and growth are in lock step, yet there is no indication that Ca^{2+} would form a prepattern before morphological differentiation, thus providing some evidence that Ca^{2+} is not acting as a morphogen in a Ca^{2+}-cytoskeleton morphogenetic mechanism. This discussion of morphogenesis in unicellular algae is shown to be relevant to higher plant morphogenesis given the deep similarities between tip growth and meristematic growth in terms of dynamics and models proposed. I conclude with suggestions for the study of pattern formation and for further research.

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Acknowledgments

In the course of this work I received the help of several persons. Foremost, my supervisor Lionel G. Harrison, who gave me all the freedom I needed and showed a constant interest in my work. My gratitude goes also to my committee members, Leah Edelstein-Keshet and Jack Maze. They enlightened me with their suggestions and mostly by showing me, with their own work, what science ought to be. I would also like to thanks:

- Dr. D. Mandoli (Botany, University of Washington) for providing *Batophora* and *Acetabularia* cells as well as many valuable ideas.

- Dr. L. Oliveira (Botany, UBC) and his Ph.D. student Lilian Alessa for sharing with me their time and extensive knowledge of cell biology.

- Dr. T. Snutch (Zoology, UBC) and his group members for providing an easy access to their micropipette puller and getting me started with my injection work.

The technical help of Angie, Audrey, Elizabeth, and Ika as well as financial support under the form of a NSERC postgraduate scholarship have been greatly appreciated. Finally, I'm indebted to David Holloway who, more than anyone else, made my transition from French to English culture a smooth and joyful experience and also introduced me to the realm of physical chemistry.

Chapter 1

Introduction

The Dasycladales order comprises 11 extant genera of unicellular green algae inhabiting shallow, protected lagoons of warm seas. The different species elongate by tip growth to reach lengths of 4 to 5 cm in some cases (e.g., *Acetabularia*, *Cymopolia*). The differentiation is under the control of a single nucleus located in the anchoring basal part, the rhizoid. The Dasycladales, and particularly the genus *Acetabularia*, were introduced to cell biology by Joachim Hämmerling more than 60 years ago (Hämmerling, 1931). Since then, they have rapidly grown in popularity for a series of reasons (Berger *et al*, 1987):

1) they are large unicellular organisms.

2) the single nucleus can be located and removed easily leading to enucleated cells.

3) they have a high regeneration capability.

4) they grow well in artificial culture conditions.

5) they show a complex morphogenesis.

All these characteristics combine to make the Dasycladales a very valuable tool for cell biology in general and for morphogenesis in particular.

1.1 Life cycle of *Acetabularia*

The life cycle of *Acetabularia acetabulum* (L.) Silva may span two or three years in the natural habitat (Woronine, 1862; de Bary and Strasburger, 1877) but is reduced to approximately six months in culture and can be shortened to three months under especially favorable conditions (D. Mandoli, personal communication). The full cycle is made of three main differentiation phases (Bonotto and Kirchmann, 1970; Figure 1). The first one, *cell differentiation*, begins with the fusion of two gametes to form the zygote which later differentiates into a rhizoid, a stalk, several vegetative whorls (hair whorls) and finally a reproductive whorl (cap). When the cap is fully

developed, the single nucleus located in the rhizoid divides and the secondary nuclei migrate to the cap. The second phase, *cyst differentiation*, is the formation of a cyst around each secondary nucleus within the gametophore (cap ray). The third and last phase, *gamete differentiation*, takes place within the cysts, the secondary nucleus in each cyst dividing usually 10 times to yield 1024 gametes (D. Mandoli, personal communication). The life cycle is completed when the operculum of the cysts opens, liberating the free swimming biflagellate gametes for mating. Cell differentiation can be further divided into developmental stages (Nishimura and Mandoli, 1992). These stages (zygote, juvenile, adult and reproductive) resemble those already used to characterize higher plant development (see for example Poethig, 1990). This work is concerned with the periodic production of whorls during cell differentiation. Descriptions of other stages can be found in works by Berger and Kaever, 1992 (zygote growth and differentiation of the rhizoid); Werz, 1968; Menzel and Elsner-Menzel, 1989 and 1990 (cyst differentiation).

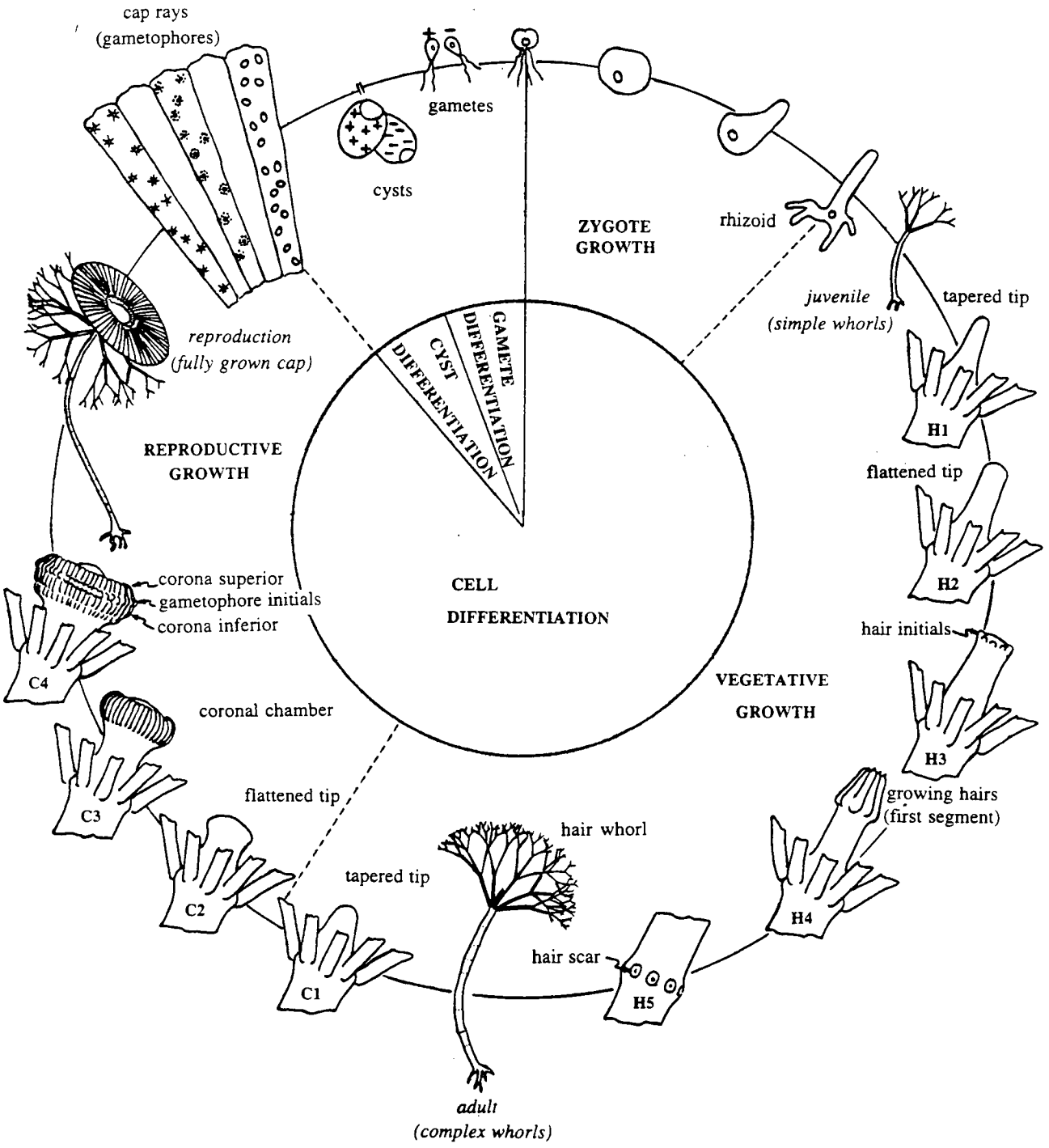


Figure 1: Life cycle of *Acetabularia acetabulum*. The full cycle last approximatively 6 months in culture. The space alloted is proportional to the time spent in each phases (based on Nishimura and Mandoli, 1992). Inner circle: differentiation phases (Bonotto and Kirchmann, 1970), outer circle: developmental phases (Nishimura and Mandoli, 1992). During cell differentiation 15 to 20 hair whorls are formed and shed (stages H1-H5) but the cap (stages C1-C4) is produced only once at the end of the phase. For a detailled staging see section 2.1.

1.2 The pattern formation viewpoint

Valet (1968) introduced an important advance in the study of Dasycladalian morphology. He noted that the field had been dominated by the study of adult morphology and departed from this long standing tradition by presenting a precise morphogenetic sequence for several genera. This work is set along similar lines as our knowledge of Dasycladalian morphogenesis is still fragmentary. This begs the question of what would be an appropriate ground on which to establish the subject. I propose, along with others (Lacalli, 1981; Harrison, 1993) the *pattern formation viewpoint*, a somewhat more precise application of Valet's morphogenetic perspective. The main concern is to pinpoint the pattern forming events and use experimental and theoretical approaches to uncover the possible morphogenetic mechanisms at work. In my view, "linear thinking", "simple causation", etc. fall short of explaining morphogenesis. Therefore the pattern formation viewpoint contrasts sharply with the perspective molecular biologists have on morphogenesis (compare for example Jürgen *et al.*, 1995). No real attempt to bridge the gap will be done within this work but the desirable task has been undertaken by others (Harrison, 1993; Green, 1994). The problems of morphogenesis are subtle and the answers are often counter-intuitive. Therefore a paradigm of how it could happen is necessary before venturing on these grounds. Three such paradigms are already available for *Acetabularia* where they ultimately take the form of mathematical models (section 1.3). Their instigators have shown that the emergence of a whorl pattern on an initially smooth tip can be explained within their paradigm and, additional work is being done to refine the models so as to match the details of the observed morphogenesis. This "theoretical and computational testing" is not the subject of this thesis. My intent is to compare the biological assumptions behind the models to what is now known about whorl formation in the Dasycladalian algae. This "experimental testing" can be approached in two ways -

1) Structural approach: one can look for and study the molecules and chemical reactions involved in the patterning mechanism.

2) Dynamical approach: one can look for and study the factors (chemical and physical) involved in the regulation of the patterning mechanism without necessarily locating any molecules or characterizing any chemical reactions.

These two approaches and their mutual interaction are best illustrated with the cybernetic concepts of *black box*, system structure and system behaviour (Calow, 1976; Figure 2). Following this analogy, the black box would contain the unknown system (i.e., the pattern forming mechanism), the input and the output would be the states before pattern formation (unpatterned state) and after pattern formation (patterned state) respectively. In this context, the first experimental approach (structural) would be a direct attempt to shed some light on a specific region of the black box by looking for molecules involved; the second experimental approach (dynamical) would involve a manipulation of some physical or chemical factors (e.g., for the Dasycladales algae: temperature, concentration of different ions in the growth medium, light quality and intensity, osmotic potential, etc.) to see their influence on the output of morphogenesis (final shape). The two approaches are mutually interacting, any new insight on the regulation process from the dynamical approach might, for example, suggest a new region of the black box wherein to look for specific molecular components of pattern formation. On the other hand, the discovery of new chemical components might suggest a new paradigm for the regulation of pattern formation and specify new factors to test.

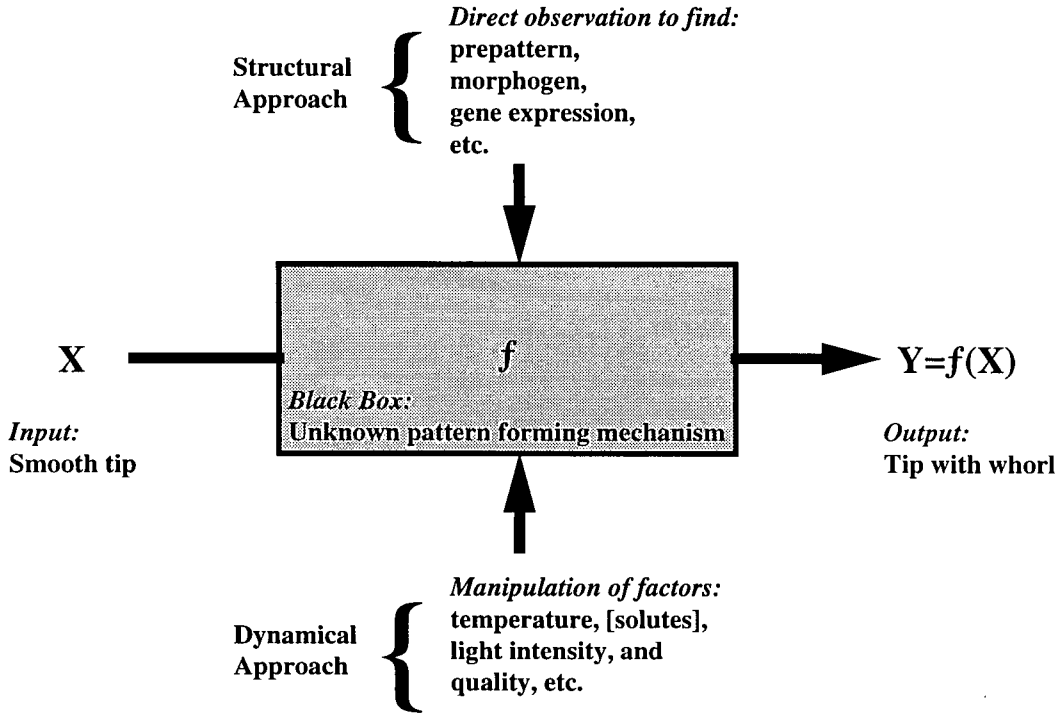


Figure 2: Structural and dynamical approaches of morphogenesis. The structural approach is concerned with locating molecules or any structural evidences for the morphogenetic mechanism. The dynamical approach is concerned with the dynamical properties of the morphogenetic mechanism.

There is no doubt that the first approach is dominant in biology at the present time, principally because of the widespread belief that the discovery of a molecule or a structural characterization is more factual than the underpinning of a chemical or physical factor controlling the process. Both types of knowledge are important in relation to morphogenesis and hence will be used in this work.

How do these ideas apply to whorl formation in the Dasycladales? A first step would be to pinpoint what is regulated during whorl formation. The development of five fingers in the human hand is an example of a precisely regulated process. For several Dasycladales species, it was found that in a constant environment, what is regulated in the whorl is not the number of laterals but the spacing

between these laterals at the time of their initiation (Harrison *et al.*, 1981). Therefore, the pattern forming mechanism at work doesn't strive to "count" a specific number of laterals but strives to space them in a constant way. As a consequence, the number of laterals (n) in a whorl is proportional to the diameter (d) of the whorl so as to provide a constant spacing ($\lambda_s \approx \pi d/n$, Figure 3).

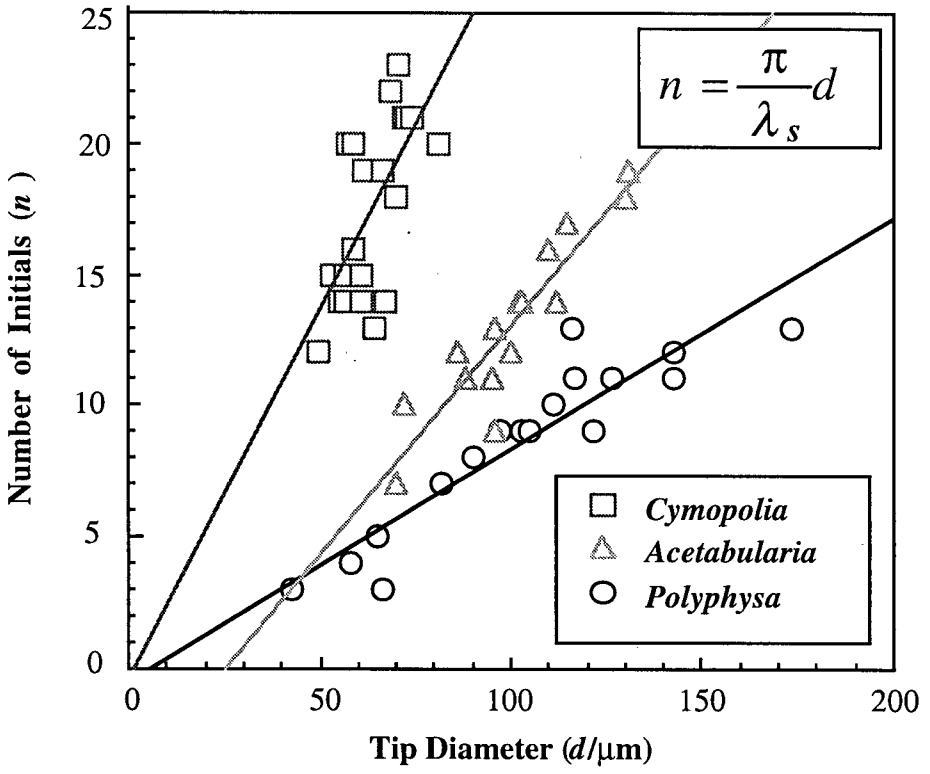


Figure 3: Number of hairs (n) versus tip diameter (d) at whorl initiation. Regression lines are - *Cymopolia van bosseae* (reproductive whorl): n = -0.44 + 0.28 d , R^2 = 0.444 (unpublished data collected by L. G. Harrison), *Acetabularia acetabulum* (vegetative whorl): n = -4.36 + 0.17 d , R^2 = 0.840 (data from Harrison *et al*, 1981), *Polyphysa peniculus* (vegetative whorl): n = -0.47 + 0.09 d , R^2 = 0.847 (this author). The slopes (approximately π/λ_s) are inversely proportional to the spacing (λ_s). The linear relation for each species is indicative of a constant spacing for given culture conditions (in this case, T ≈ 20 °C and $[Ca^{2+}]$ ≈ 7 mM -> *Cymopolia*: λ_s ≈ 11.2 μm; *Acetabularia*: λ_s ≈ 18.5 μm; *Polyphysa*: λ_s ≈ 34.9 μm).

In section 1.4 it will be shown how the idiosyncrasies of the three models and the constant spacing can be used to test specifically the structural and dynamical aspects of whorl formation. This discussion needs to be preceded by a short introduction to tip growth, the primary growth mechanism in *Acetabularia* and other Dasycladales (Puiseux-Dao, 1965).

1.3 Tip growth

Tip growth occurs in several cell types (fungal hyphae, root hairs, pollen tubes, and certain algae). A great number of ideas have been advanced to explain the control of tip growth (see the references below). Explanation for this plurality of ideas must be sought in either the diversity of growth mechanisms in plant and fungal cells or the fundamental complexity of the process itself. Even if I intend to focus on pattern formation, growth remains an integral part of morphogenesis. It is therefore worthwhile to summarize the different ideas about tip growth to eventually show their close connection with pattern formation of whorled structures.

Polarity is often refered to as the leading principle of morphogenesis (Nakazawa, 1989). Tip growth is a clear expression of polarity within a cell while whorl formation is a more complex example where uniaxial polarity (stalk growth) is transformed into multiaxial polarity (whorl growth). Polarity, whether chemical or physical in nature, is therefore at the very heart of morphogenesis. The initiation of polarity and its maintenance is what needs to be explained to understand tip growth and morphogenesis.

Two observations appear to be shared by most tip growing organisms. First, the growing tip shows a high Ca^{2+} concentration as reported in *Acetabularia* (section 4; Reiss and Herth, 1979; Harrison *et al*, 1988), pollen tubes (Reiss and Herth, 1978), *Micrasterias* (Meindl, 1982) and fungal hyphae (Reiss and Herth, 1979). Second, Golgi vesicles have been shown to be vectorially transported to the tip in such systems as fungal hyphae (Steer, 1990), root hairs (Sievers and

Schneff, 1981), pollen tubes (Picton and Steer, 1982), and *Chara* rhizoids (Sievers and Schneff, 1981). The role of these vesicles is clear; they provide the tip with the necessary material (cell membrane, cell wall precursors) to sustain growth. The involvement of Ca^{2+} appears to be more subtle but the ion is often taken as the regulator of tip growth through its interaction with the cytoskeleton and a variety of enzymes.

One significant advance has been made with the discovery that the arrival of vesicles to the tip is necessary but not sufficient to explain growth. Such evidence was provided by Kiermayer's experiments on the growth of *Micrasterias* in hypertonic solution (Kiermayer, 1964). He observed that the decrease in turgor pressure resulting from the hypertonic solution stopped growth completely but didn't inhibit the secretion of wall components by the Golgi vesicles. After a few minutes the cells showed a characteristic wall thickening in the regions where growth would have occured in normal conditions. Some authors obtained similar results by subjecting pollen tubes to ionophore A23187 (Picton and Steer, 1982).

It appears from these observations that an element is missing to fully account for the forward movement of the tip. Since normal wall deposition, but no growth, can be observed without a reduction of turgor pressure; and, alternatively, normal growth can occur without any measurable turgor presssure (Harold *et al.*, 1995), an alternative to turgor pressure is required to explain growth. Three such explanation have been proposed (Steer and Steer, 1989). First, the wall itself could control its own extension. The wall could be secreted in a "plastic state" allowing for growth but then stiffen as it is incorporated in the existing wall. Second, an alternation between the secretion of stiff wall and local lysis could account for the growth of a specific region. Third, the wall could be always fairly fragile at the tip region and the cytoskeleton would provide the necessary support and as such regulate growth (Harold et al., 1995; Heath, 1995). The latter explanation is most favored now.

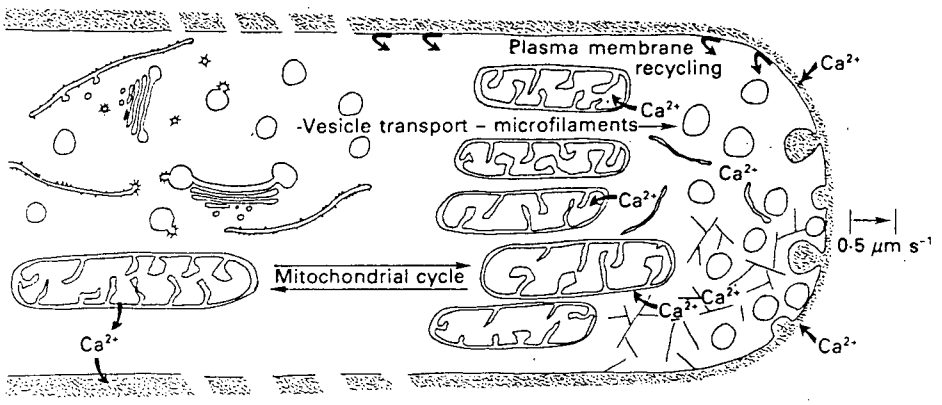


Figure 4: Tip growth. Diagram of a pollen tube section, a typical tip growing cell. Two distinctive regions are observed: a region densely filled with Golgi vesicles at the apex of the cell and a cluster of mitochondria in the subapical region. The vesicles provide material for the growth of the wall while the mitochondria might be involved in the regulation of the concentration of free cytosolic Ca^{2+}. (From Steer and Steer, 1989).

1.4 The three proposed pattern forming models

Three models have been put forward to explain whorl formation in *Acetabularia*. Surprisingly, these three models have few characteristics in common. Each model relies on a different kind of mechanism (mechanical, chemical and mechano-chemical) and attributes organization of the whorl to a different cellular unit (cell wall, cell membrane, cytoskeleton and cortical cytosol). I have chosen to give a graphical illustration of each model's dynamics and an overview of their biological assumptions rather than an extensive mathematical treatment for which the reader should consult the original references as cited below.

Martynov's model

Martynov (1973, 1975) suggested that the cell wall could drive whorl formation or, as he said it, the cell wall would act as a "spatio-temporal coordinating morphogenetic instrument". He recognized that the question addressed was in many ways more complex than his treatment but he saw a great value in showing that the cell wall alone could account for the phenomenon observed. The model is based on the idea of loss of mechanical stability (Martynov, 1973, 1975). It has long been observed that before the initiation of a whorl, the cell tip shifts from apical growth to lateral growth. Martynov used this fact to argue for a loss of mechanical stability of the tip when its width reaches a threshold. At that time the turgor pressure initiates a series of folds on the previously smooth tip. To my knowledge, Martynov has never provided a complete mathematical representation of the dynamics of his model. The only equations available are approximations of important parameters. At the heart of his model lies the approximation for the critical pressure value (P_{cr})(Martynov, 1975):

$$P > P_{cr} \approx \frac{E\delta^2}{\sqrt{3}b^2\left(\frac{a^2}{2b^2}-1\right)} \qquad (1)$$

where E = Young's modulus
P = turgor pressure
a = major semi – axis of the cell tip (diameter / 2)
b = minor semi – axis of the cell tip
δ = wall thickness

The increase in diameter ($2a$) lowers P_{cr}, that is, as the tip broadens the stresses in it are increased. When P_{cr} decreases below the turgor pressure value (P) there is loss of stability and formation of folds on the tip (Figure 5). The spacing (λ_s) between these folds is given by the following two approximations (Martynov, 1975):

$\lambda_s = 2\pi a / n \approx 0.54\ \pi\ (a\delta)^{1/2}$ (for the hair whorl) (2a)

$\lambda_s = 2\pi a / n \approx 1.29\ \pi\ \delta$ (for the cap) (2b)

In the likely event where the wall thickness (δ) is nearly constant, Equation (2a) would suggest that λ_s is not, as shown earlier, independent of the tip size as measured by a or d (a=d/2). Simple algebraic manipulations on Equation (2a) show that the number of initials (n) would be proportional to $a^{1/2}$ or $d^{1/2}$ instead of being directly proportional to d. Looking back at Figure 3, the measurements made on species from three different genera provide little evidence for such a relationship. Martynov (1975) himself published data correlating the number of initials n with a/δ, the inverse of what he called the *tip rigidity* (δ/a). Given the strong dependency of n on d, these data provide a poor test of the effect of wall thickness (δ) on the number of initials or the spacing (λ_s). Therefore the crux of the buckling mechanism, i.e., the dependency of spacing on wall thickness, has yet to be tested properly.

The initial amplitude (e) of the folds is given by (Martynov, 1975):

$$e \approx \delta / 3 \qquad \text{(for the hair whorl)} \qquad (3a)$$

$$e \approx \delta / 40 \qquad \text{(for the cap)} \qquad (3b)$$

Given that the wall thickness rarely exceeds 10 μm, the amplitude of these folds is very small compared to the size of the tip itself (ranging from 50 μm to 300 μm). Irrespective of their size, the folds and the accompanying prepattern of strain would form a template for the subsequent growth of the appendages. The prepattern of strain could be transduced into growth via *stretch activated ion channels* (Garrill *et al*, 1993) leading to hydrolysis of the cell wall, relaxation and growth (Figure 6a). Ca^{2+} ions could be involved in the control of growth leading to the loss of stability and buckling; but, more importantly, they could act as second messengers in the transduction of the mechanical prepattern into growth (Knight *et al*, 1995).

Harrison's model

Harrison's model is based on Turing's reaction-diffusion theory (1952) which showed that specific interactions between two molecules (X and Y morphogens) can lead to a pattern of high and low concentration of these molecules from an initially homogeneous system (Figure 7). Most likely, one or both of these morphogens would be integral membrane proteins (Harrison, 1996). One of the simplest reaction-diffusion systems is the Brusselator (Prigogine and Lefever, 1968). It is represented by the following set of differential equations:

$$\begin{cases} \dfrac{\partial X}{\partial t} = aA - bBX + cX^2Y - dX + D_X\nabla^2 X \\ \dfrac{\partial Y}{\partial t} = \qquad bBX - cX^2Y \qquad + D_Y\nabla^2 Y \end{cases} \tag{4}$$

where X = concentration of chemical X (activator)
Y = concentration of chemical Y (inhibitor)
A, B = concentration of chemicals A and B
a, b, c, d = rate constants
D_X, D_Y = diffusion coefficients

The model is composed of two such reaction-diffusion systems in series, the output X of the first serving as an input A of the second. The first system (stage 1) accounts for the transition from a growth maximum at the apex to a subapical growth annulus. The second system (stage 2) feeds on this annular region and breaks it into a whorl pattern. The two stages are required for theoretical and empirical reasons. First, a single reaction-diffusion system operating freely on a hemispherical tip leads to a whorl pattern only if the tip is small (number of peaks smaller than 5) while a larger tip will usually lead to a random distribution of the morphogen peaks (Harrison *et al*, 1981, 1988). To account for the large whorls the second reaction-diffusion system must be constrained to an annular region generated by the first stage. The two stages are also empirically motivated since various treatments will switch off whorl formation (stage 2) without stopping tip growth (stage 1), showing their relative independence. Examples of such treatments are exposure to red light only

(Schmid, 1987), exposure to ionophore A23187 (Goodwin and Pateromichelakis, 1979), exposure to high or low Ca^{2+} concentration (Goodwin *et al*, 1983).

The spacing (λ_s) provided by the second stage is given for a Brusselator by (Harrison and Hillier, 1985):

$$\lambda_s = 2\pi\left(\frac{D_X D_Y}{(a^2bc/d^2)(A^2B)}\right)^{1/4} \quad (5)$$

and all two-morphogen reaction-diffusion mechanisms give dependences of λ_s on inverse powers of input concentration. The effect that these parameters have on λ_s has been tested in this lab. By varying the temperature at which the cells are grown, one can influence the diffusion coefficient (D_X and D_Y) and the rate constants (a, b, c, and d). A rise in temperature increases the reaction rate to a greater extent than the chemical diffusion leading to an overall decrease in spacing (Harrison *et al.*, 1981). This prediction from the model has been verified experimentally (Harrison *et al.*, 1981). The role of Ca^{2+} ions as precursors (i.e., A or B) for the morphogens can also be experimentally tested by varying the Ca^{2+} concentration of the culture medium ($[Ca^{2+}]_e$). The results (Harrison and Hillier, 1985; also reproduced by Goodwin *et al*, 1985) showed a decrease of λ_s as $[Ca^{2+}]_e$ increased and can therefore be easily accounted for by reaction-diffusion theory. The transduction of the morphogen prepattern into growth could be achieved in several ways (docking proteins for Golgi vesicles, ions channels or transmembrane signal transduction proteins) (Figure 6b).

Goodwin's model

Goodwin and Trainor (1985) presented a model for whorl formation in *Acetabularia* involving an interaction between the cytoskeleton and cytosolic calcium. This interaction affects the visco-elastic state of the cytoplasm and can therefore be used to generate a field of stresses acting mechanically on the cell membrane and eventually on the cell wall (Figure 8). The interaction is mechano-chemical. First, the mechanical state of the cytoplasm influences the free cytosolic calcium. If the

system is strained (stretched), the free calcium concentration increases whereas if the system is compressed the free calcium concentration decreases. Second, the calcium influences the mechanical state of the system; an increase in free calcium induces a solation of the cytoplasm (decreasing viscosity) and influences the elastic modulus of the cytoplasm in a complex fashion. Contrary to the previous two models, Ca^{2+} ions are involved directly as morphogens in this model. The interaction can be expressed by the following system of differential equations (Goodwin and Trainor, 1985):

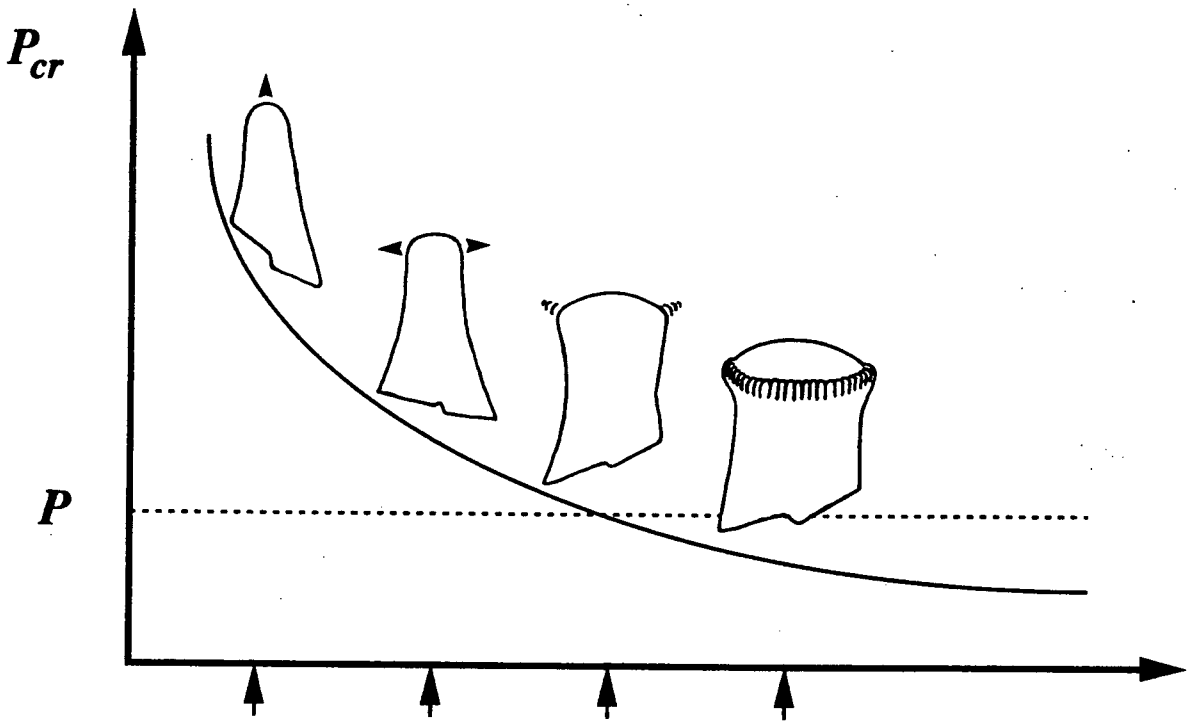
$$\begin{cases} \rho\dfrac{\partial^2\bar{\xi}}{\partial t^2} = \mu\nabla^2\bar{\xi} + (\lambda+\mu)\nabla(\nabla\cdot\bar{\xi}) + \eta\nabla^2\dfrac{\partial\bar{\xi}}{\partial t} + (\zeta+\eta/3)\nabla(\nabla\cdot\dfrac{\partial\bar{\xi}}{\partial t}) \\ \qquad -c\dfrac{\partial\mu}{\partial\chi}\nabla\chi - R\bar{\xi} + \text{second order terms} \\ \dfrac{\partial\chi}{\partial t} = \left(a + a_{ij}\dfrac{\partial\bar{\xi}_i}{\partial x_j}\right)(K-\chi) - k_1(\beta+\chi)\chi^n + D_\chi\nabla^2\chi \end{cases} \tag{6}$$

where $\bar{\xi}$ = displacement of a unit element of cytogel from the equilibrium position
χ = concentration of free Ca^{2+}
x = spatial variable
μ, λ and ζ, η = constants of elasticity and viscosity respectively
D_χ = Ca^{2+} diffusion coefficient
k_1 = rate constant
a, a_{ij} = coupling coefficients
c = initial strain
R = restoring force
β = Ca^{2+} binding sites available
K = total intracellular [Ca^{2+}]

The equation for the spacing hasn't been written explicitly but computations have shown the influence of a series of parameters on λ_s (Brière, 1994):

$$\lambda_s = f^+ (c^{-1}, K^{-1}, a_{ij}^{-1}, D_\chi, \text{elastic modulus } [\lambda,\mu], R^{-1}) \tag{7}$$

All these parameters have a physical/biological meaning but most of them are not accessible to measurements. It is therefore not surprising that the authors have not submitted the model to direct experimental testing. The experiments on the effect of $[Ca^{2+}]_e$ on λ_s come closest to such a test if one supposes that the total intracellular $[Ca^{2+}]$ (i.e., K) is directly affected by the extracellular Ca^{2+} pool. If this is the case, the Ca^{2+} data presented for the previous model would fit equally well Goodwin's model. Goodwin and Trainor (1985) proposed that the strain prepattern could be transduced into growth by stretch activated ion channels. This would lead to a secretion of H^+ ions inducing local lysis of the cell wall (Figure 6c).



$$P > P_{cr} \approx \frac{E\delta^2}{\sqrt{3}b^2\left(\frac{a^2}{2b^2} - 1\right)}$$

Figure 5: Schematic description of the dynamics of Martynov's model. The curve and the equation describe the evolution of the critical pressure (P_{cr}) as the tip radius (a) increases. The side growth occuring in the subapical region will drive P_{cr} under the turgor pressure value (P) where the mechanical stability of the cell wall will be lost and buckling will occur. The first pattern forming event, that is, the transition from apical growth to side growth, hasn't been made explicit by Martynov.

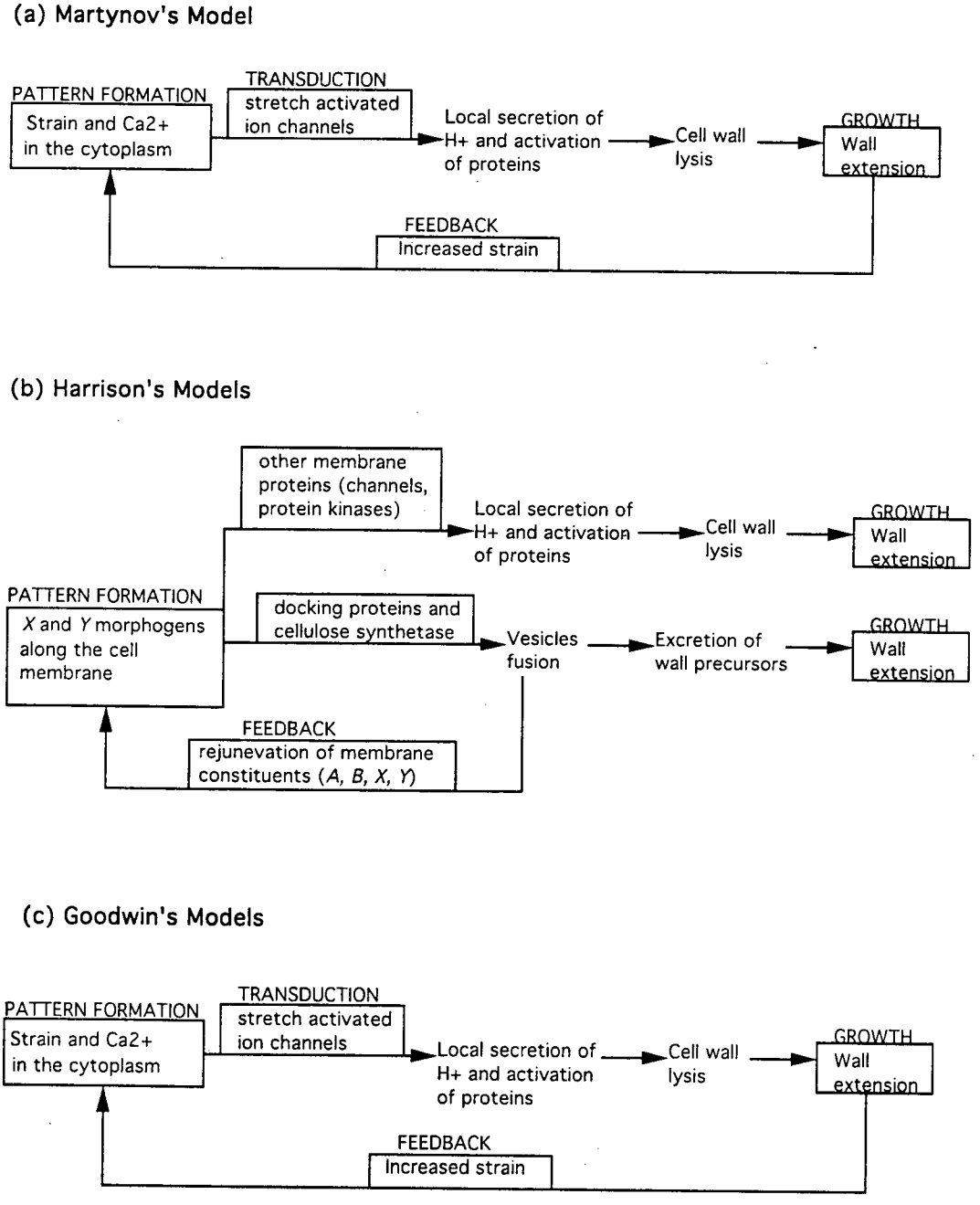


Figure 6: Relationship between pattern formation and growth. For morphogenesis to occur, pattern formation and growth must interact. This figure provides possible feedback loops for the different morphogenetic mechanisms.

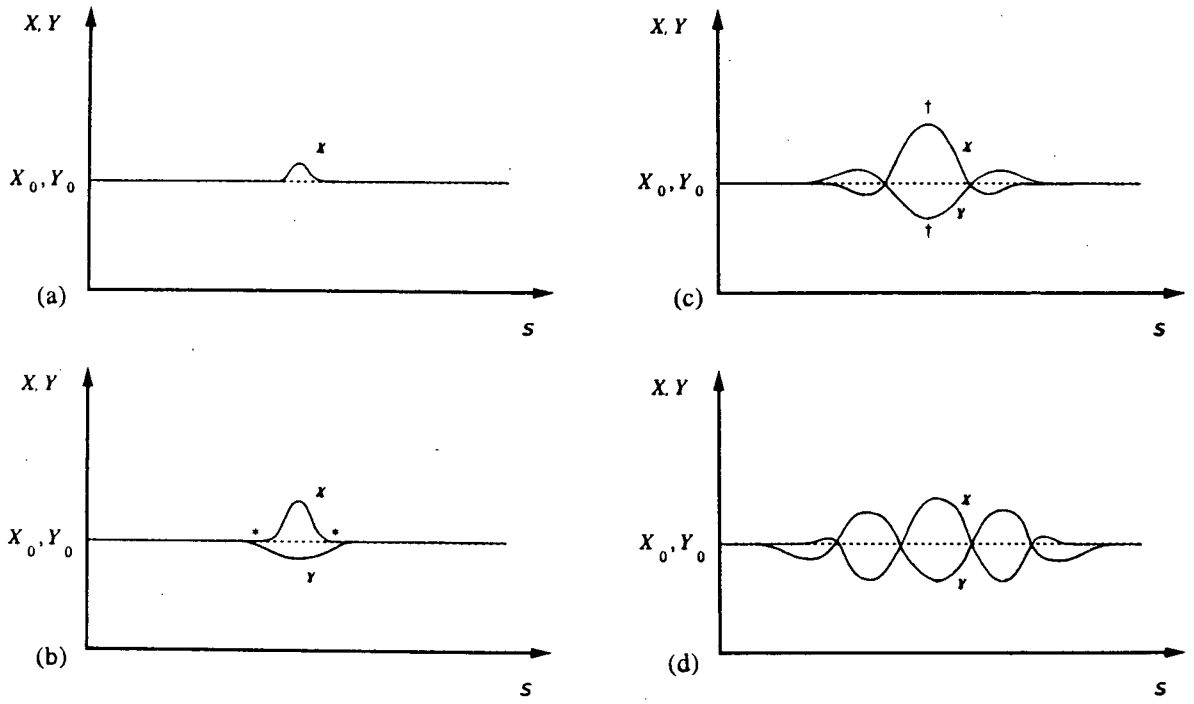


Figure 7: Schematic description of the dynamics of a Brusselator mechanism. Each graph represents the concentration of the two morphogens (X, Y) along one spatial dimension (s). (a) A small perturbation from the homogeneous steady state (X_0, Y_0) of one of the morphogens (here X) leads to an increase in X (X catalyzes itself) and a depletion of Y (X uses up Y for its own catalysis). (b) Since Y diffuses faster than X, the trough in Y distribution will enlarge faster than the peak in X distribution (*). In the periphery, where $X=X_0$ but $Y<Y_0$, the production of X is decreased (Y is needed for the steady production of X). (c) The decrease of X below X_0 enables a rise of Y above Y_0. At the same time the initial X peak and Y trough approach new steady state values (†).(d) Further propagation in the system will create a series of peaks and troughs in the X and Y distributions. Each peak could drive the differentiation of one whorl initial. (Modified from Maynard Smith, 1968).

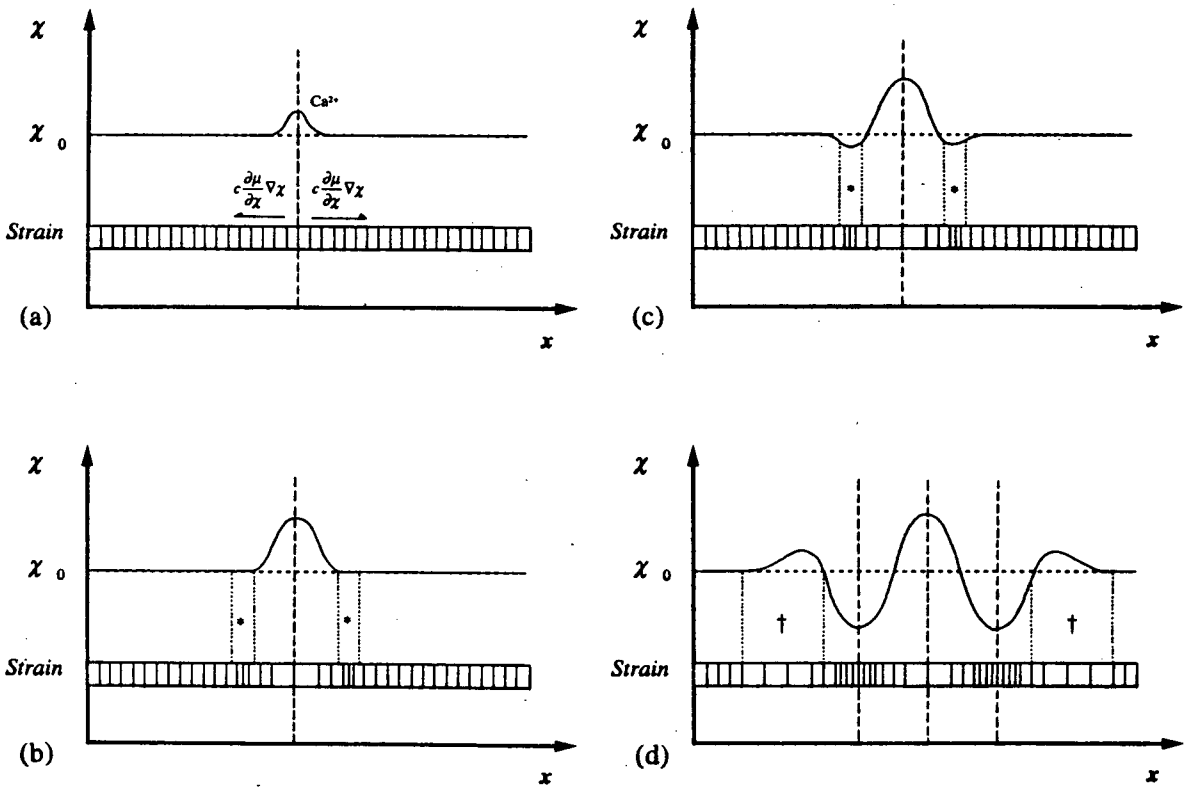


Figure 8: Schematic description of the dynamics of Goodwin's model. Each graph gives the free Ca^{2+} concentration (χ) and the distribution of strain along one spatial dimension (x).(a) The gradients of free Ca^{2+} brought about by a small perturbation from the homogeneous steady state concentration (χ_0) induce opposite forces on the two sides of the Ca^{2+} peak. (b) As a result, the central cytoplasmic region will be stretched, while the periphery will be compressed. Because mechanical signals are fast and diffusion is slow, regions (*) will develop where the cytoplasm is compressed while $\chi=\chi_0$.(c) Since the mechanically compressed cytoplasm offers more binding sites for free Ca^{2+}, χ will drop below χ_0. The low χ value will affect the mechanical state of the cytoplasm in that region and increase the local compression up to a certain point. (d) The compression will stretch the cytoplasm in the periphery (†) reducing the number of binding sites available. Therefore χ will rise and propagate the initial perturbation across the system. In this model, the concentration of strain and free Ca^{2+} in regularly spaced regions would eventually lead to the formation of whorl initials.

Summary of idiosyncrasies

These widely different models are a glaring expression of how little we know about morphogenesis. The numerous discrepancies make a strong call for open-mindedness and more experimentation. Given that the models have the theoretical requirements to account for whorl formation as shown by diverse computations, the next step is to test them experimentally. Based on the two approaches exposed in section 1.2 one can either test the structural idiosyncrasies, i.e., look for the location of the prepattern (cell wall, cell membrane, cytoplasm,) and its nature (molecules, strain)(Figure 9, column 2, 3 and 4) or test the dynamical idiosyncrasies by looking at the effect of physical and chemical factors on λ_s (Figure 9, column 5). Figure 9 is central to this work as most of my energy went into trying to test some of these idiosyncrasies (specifically columns 2, 3 and 4).

	1) Type of Model	2) Nature of the prepattern	3) Prepattern location	4) Possible role of Ca2+	5) $\lambda=f(x)$
Martynov	Mechanical (equilibrium)	Strain	Cell wall	Second messenger (transduction)	$f+$ (δ), wall thickness $f-$ (a), tip semi-axis
Harrison	Chemical (kinetic)	X and Y morphogens	Cell membrane	Precursor to one morphogen	$f+$ (DX, DY), X,Y diff coefficient $f-$ (a, b, c, d), rate constants $f-$ (A, B), [A] and [B]
Goodwin	Mechano-chemical (kinetic)	Ca2+ and Strain	Cytosol	Morphogen	$f-$ (c), initial strain $f-$ (aij), coupling coefficient $f+$ (λ,μ), elastic modulus $f+$ ($D\chi$), Ca2+ diff. coefficient $f-$ (K), [Ca2+] in the cytosol $f-$ (R), restoring force

Figure 9: Summary of the models' idiosyncrasies. Column 1 is a classification based on Harrison, 1987 and 1993; columns 2, 3 and 4 are particularly useful for the structural approach of morphogenesis; column 5 corresponds to the dynamical approach of morphogenesis. $f+$: positive function (i.e., $df/dx>0$), $f-$: negative function (i.e., $df/dx<0$).

Chapter 2

Developmental sequence

Although the morphology of several Dasycladalian species has been the subject of extensive description (Berger and Kaever, 1992) there are few instances where a definite morphogenetic perspective has been taken. In this section, I present a detailed description of whorl formation in *Acetabularia*, a comparison with selected genera, and a discussion of the normal and teratological variations observed. The approach is mainly descriptive, but explanation for some of the features observed will be provided in section 3. This work has no claim of being completely new though some of its content is potentially unknown to the practitioners of the field.

For these observations I had access to cultures of *Acetabularia acetabulum* (L.) Silva and *Polyphysa peniculus* Agardh grown in artificial sea water (Shephard's medium, Shephard, 1970) at a temperature of 20 °C and a 12:12 h light/dark cycle. Specimens of *Batophora oerstedi* Agardh and a second cell line of *A. acetabulum* (Aa0006) were also kindly provided by Dr. D. Mandoli (University of Washington). The latter was grown in modified Müller's medium (Müller, 1962; modified by Schweiger *et al.* , 1977) enabling a comparison between two distant cell lines grown in different culture conditions.The information presented is based on observation of cell wall under a standard compound microscope. The freehand wall sections were cleaned of their cytoplasmic content with a bleach solution (1% $NaOCl:H_2O$) and were preserved in a water-glycerine solution. When needed, the cells were stained with a diluted toluidine blue solution. The photographs were taken on 200 and 400 ASA Fuji color negatives with exposure of 1/15 second. Unless otherwise mentioned, the drawings are based on my own photographs and microscope observations. Though some attention has been given to preserve the proportions, their main purpose is to underline features not easily conveyed by the photographs available.

2.1 Whorl formation in *Acetabularia*

Comprehensive overviews of whorl formation in *Acetabularia* were provided by Solms-Laubach (1894), Howe (1901) and Valet (1968). I supplement the previously known facts in two ways; first, consistent with the perspective adopted, the whole process of whorl formation is subdivided into stages characteristic of the different pattern forming events; second, the early stages of *whorl formation* are resolved more clearly thus adding to the pioneering work of Werz (1965, see *Stage 3: wall lysis* in this section).

Despite their very different external aspects, the vegetative whorl (hair whorl) and the reproductive whorl (cap) share a common structure. This shared structure is easily explained given that both whorl types go through the same four stages of morphogenesis, the cap involving an additional fifth stage. These stages are preserved for the two cell lines studied (our own Bev line and Mandoli's Aa0006 line) even if the overall morphologies show slight differences. They are treated in their order of appearance (Figure 10).

Stage 1: Apical growth

Apical growth dominates the morphogenesis of *Acetabularia* from the first appearance of polarity in the zygote to the growth of the cap, and the resumption of growth after wounding. Only temporarily will apical growth be stopped to give place to the whorl-forming stages of morphogenesis. This stage is probably the most primitive and as such, the most resilient to variation in growth conditions (e.g., low and high calcium concentration, Goodwin *et al*, 1983). Morphologically, an apically growing tip ranges from a tapered shape to a dome shape. Careful observation reveals that the apex is densely filled with vesicles (Schmid *et al.*, 1987) as observed in many tip growing cells (see section 1.3). Finally, it has been demonstrated for cells grown in red light that the pattern forming event at the origin of the transition from apical growth to the following stage requires blue light (Schmid *et al.*, 1987).

Stage 2: Side growth

A flattening tip is the first morphological indication of whorl initiation. The pattern forming event involves redirecting apical growth to a subapical annulus of growth. The lateral bulging of the subapical region is usually minimal when a vegetative whorl is initiated but a strong lateral ridge can be seen at the initiation of a reproductive whorl (Figures 10, 22a, 23a, 24a,). A close look at this actively growing region of the reproductive whorl reveals that it is associated with a thickening of the cell wall (Figure 12a). This observation suggests an intense secretion of wall precursors to sustain growth. Kiermayer (1981) made similar observation in *Micrasterias* where cells placed in a slightly hypertonic solution show a local thickening of the wall in actively growing regions. It isn't known if this thickening forms a continuous ring or if it is formed of broken regions already prefiguring the position of appendages in the whorl.

Stage 3: Wall lysis

As the tip is still broadening, the first structural cue of the location of the appendages manifests itself as a punctate lysis of the inner wall (Figures 11a and 12b,c). This was reported first by Werz (1965); but, as far as I know, the observations were made fairly late in development (see Figure 12d,e). At inception, the lysis pattern is constrained to a narrow line circling the broadened tip precisely in the region of greatest curvature. The observation shows that the lysis points appear first as very fine notches in the inner wall. These notches then extend principally along the cell axis to form small chambers in the cell wall (Figure 12d,e). The spacing in the cap is much smaller than in the hair whorl (4 to 5 μm compared to 15-25 μm). The transition from the presumably uniform ridge region of Stage 2 to the lysis prepattern of this stage is, in my view, the key morphogenetic event in the Dasycladales, yet it has been the object of very little research.

Stage 4: Appendage growth

The growth of the appendages follows the lysis without a marked transition and, in that sense, no further pattern formation has occurred. As the lysis chambers develop in the cell wall, little bulges

betray their presence externally (Figure 12e). The bulges assume a different shape depending on what whorl-type is produced (vegetative or reproductive), but they will, despite their different aspect, go through similar steps of differentiation (i.e., apical growth, septum formation, and branching). The apical growth of the initials is very pronounced for the vegetative whorl and fairly limited for the reproductive whorl (Figures 11c,d and 12f). Concomitantly with the apical development, a perforated septum forms slightly distal to the junction between the lateral and the central cavity. This septum divides the lateral into a proximal chamber, the *vestibule*, and a distal chamber, the *hair segment* for the vegetative whorl and the *coronal chamber* for the reproductive whorl (Figures 11d and 12f). In contrast with the cap vestibule which remains conspicuous, the hair vestibule vanishes during the ulterior development of the whorl and we owe its discovery to Valet's careful investigation (see Valet, 1968, page 79 and Table 13). The perforated septum is formed by infurrowing of the lateral wall. Externally, the infurrowing leads to the characteristic crease of the corona inferior and a slight girdle-like appearance in the hair whorl (Figures 11c,d and 12f). The septation is not complete as a pore allows cytoplasmic communication between the central cavity and the lateral appendages. The pore can be closed irreversibly by a plug secreted by the cell (Menzel, 1980). Soon after the septum has been initiated, the appendages branch. The secondary branching is similar to primary branching (i.e. whorl formation). It involves broadening of the tip, lysis, secondary initial growth, etc.(for the vegetative whorl see Figure 11c,d and Puiseux-Dao, 1965). The vegetative and reproductive whorls seem to differ with respect to the branching of their appendages. In the former the second order branches and higher order branches still form a whorl structure while the latter presents second order branches along a straight line (Figure 10). Evidence suggests that the difference is superficial. Howe (1901) and Valet (1968) observed in *A. acetabulum* that the protuberances of the corona superior are initiated in a whorl formation and they are only ulteriorly rearranged in a row as the structure supporting them, i.e., the coronal chamber, undergoes additional growth. Valet took the residual wavyness of this row as an additional proof of his assertion. I haven't been able to confirm this; but the fact that related

species (e.g. A. *caliculus*) show whorled protuberances would suggest the exactitude of their observations.

Stage 5: Gametophore growth

The growth of the gametophore (i.e., cap ray where the cysts are formed) is the last stage of morphogenesis and pertains to the cap only. The gametophore is formed only after the appearance of the protuberances of the corona superior. It is initiated as a local bulge that grows into the characteristic club shape (Figures 12f and 14a). This event subdivides the coronal chamber into a corona superior region and a corona inferior region, while the succeeding growth will tend to distort the coronal chamber (compare Figures 12f and 14a). Contrary to what has been seen in previous stages, the growth in the gametophore is not localized at the tip only and the structure lacks the perforated septum.

This staging provides useful information about the possible involvement of the three models. The location of pattern formation in the region of greatest wall curvature (Figures 11a, 12a,b) has been predicted by all models. Whether the explanation is given in terms of stress concentration or growth maximum, the theory and the observations are consistent. On the other hand, the wall thickening, the lysis and the spacing all tend to undermine wall buckling as the pattern forming mechanism. The thickening in the morphogenetic region probably increases the wall stiffness at this location, yet it is this region that would have to buckle to account for whorl formation. In the following stage, the lysis seems to precede the bulging of the wall instead of deriving from it. But given that the buckling amplitude predicted by Martynov's model is at best 3 μm, buckling might have occured without providing a clear indication of its presence. Finally, based on measurements from Figure 11a, Equations (2a) predicts a spacing of 20 μm, a very good estimate of the observed spacing of 19 μm, but similar measurements from Figure 12b,c do not support the involvement of buckling for the cap pattern formation. In this case, the prediction based on Equation (2b) (i.e., $\lambda_s \approx 48$ μm) is 12 times greater than the observed value (i.e., $\lambda_s \approx 4$ μm).

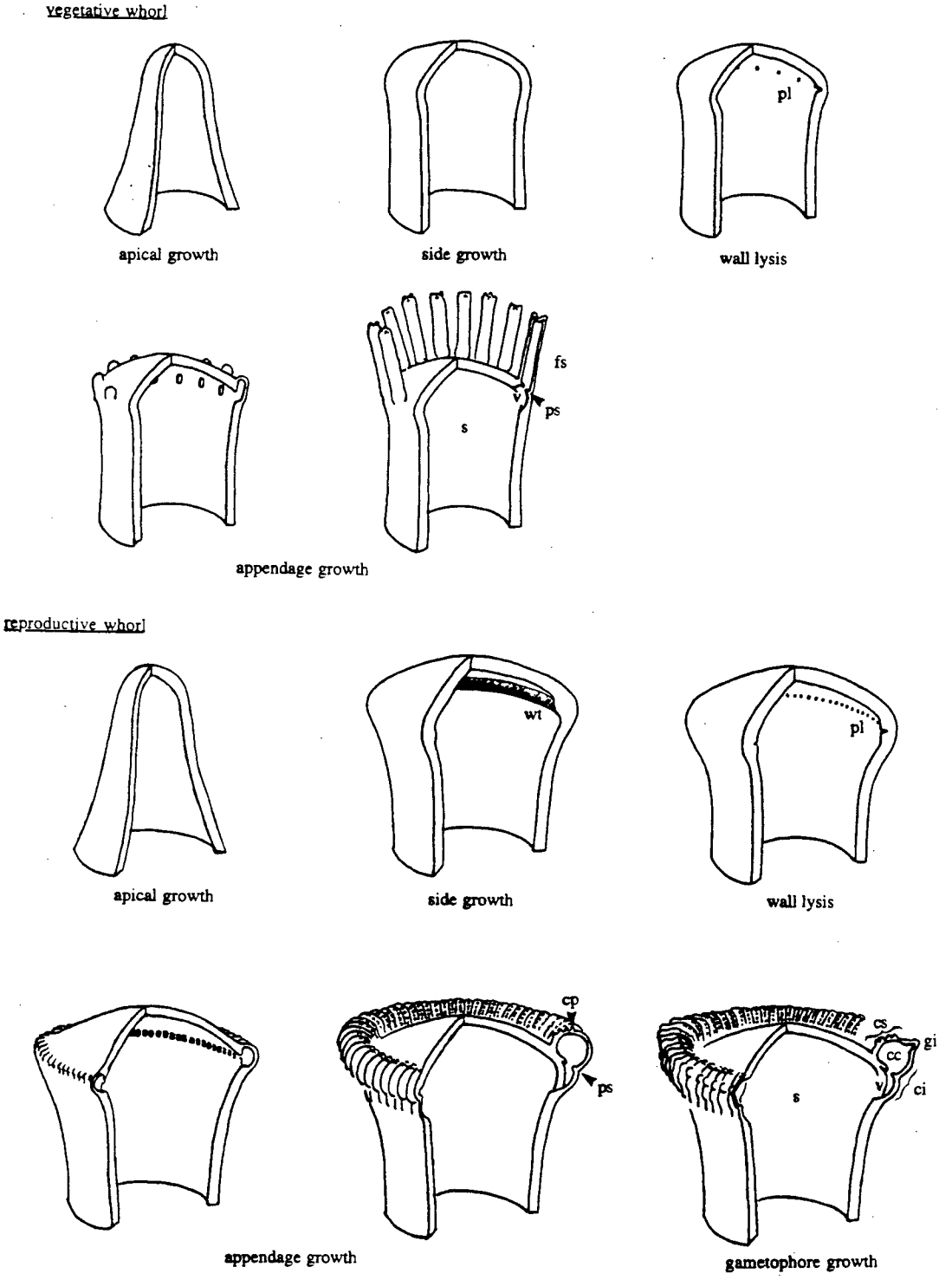


Figure 10: Morphogenetic staging of the vegetative and reproductive whorls of *Acetabularia acetabulum*. Legend- cc: coronal chamber, ci: corona inferior, cp: corona protuberance, cs: corona superior, fs: first hair segment, gi: gametophore initial,.pl: punctate lysis, ps: perforated septum, s: stalk, ss: second hair segment, v: vestibule, wt: wall thickening.

Figure 11: Critical steps in the morphogenesis of vegetative whorls. Morphogenesis of the vegetative whorl is essentially the same in all genera observed. The genus offering the best illustration of a specific structure has been choosen for this figure (a-d, *Polyphysa peniculus*, e and f, *Batophora oerstedi*). (a) Wall lysis (arrowheads). The initiation of a first whorl was aborted and would explain the deformity of the tip (bar=50µm). (b) Appendage growth. Note the initiation of the perforated septa (arrowheads)(bar=50µm). (c) Flattening of the primary hair segments and early initiation of secondary hair segments (stars). Note again the perforated septa and the slight girdling at the base of the primary hair segments (arrowheads). The septum defines a proximal chamber (vestibule) and a distal chamber (hair segment itself)(bar=50µm). (d) The secondary hair segments are differentiating following the same stages as the primary segments (bar=50µm). (e) Close-up of the perforated septa. The pores are clearly visible (arrowhead)(bar=25µm). (f) Lateral view of the septum and the pore. The thickened lips of the pore are seen (arrowheads)(bar=20µm).

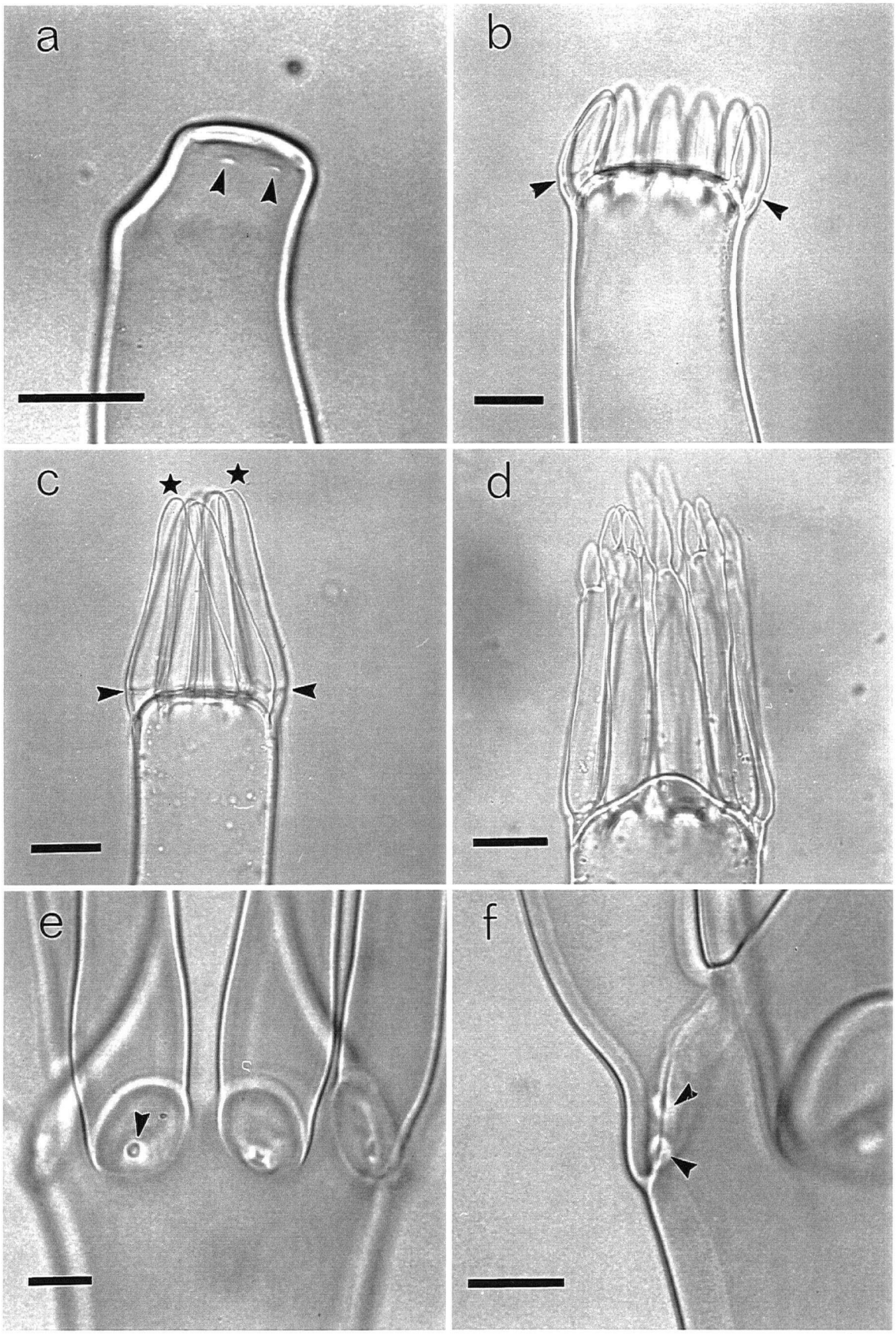


Figure 11

Figure 12: Critical steps in the morphogenesis of the reproductive whorl of *Acetabularia acetabulum*. (a) Thickening of the inner wall in the morphogenetic region (arrowheads)(bar=50μm). (b) and (c) Lysis of the inner wall showing the deep notches (b, arrowheads) and the beautifully regulated spacing (c)(bar=50μm). (d) and (e) Later stage showing the lysis chambers elongating principally toward the apex (d) and the bulging that reveals their presence externally (e)(bar=50μm). (f) Coronal chambers initiating the gametophores (stars). Note the crease produced by the perforated septa (arrowheads)(bar=50μm).

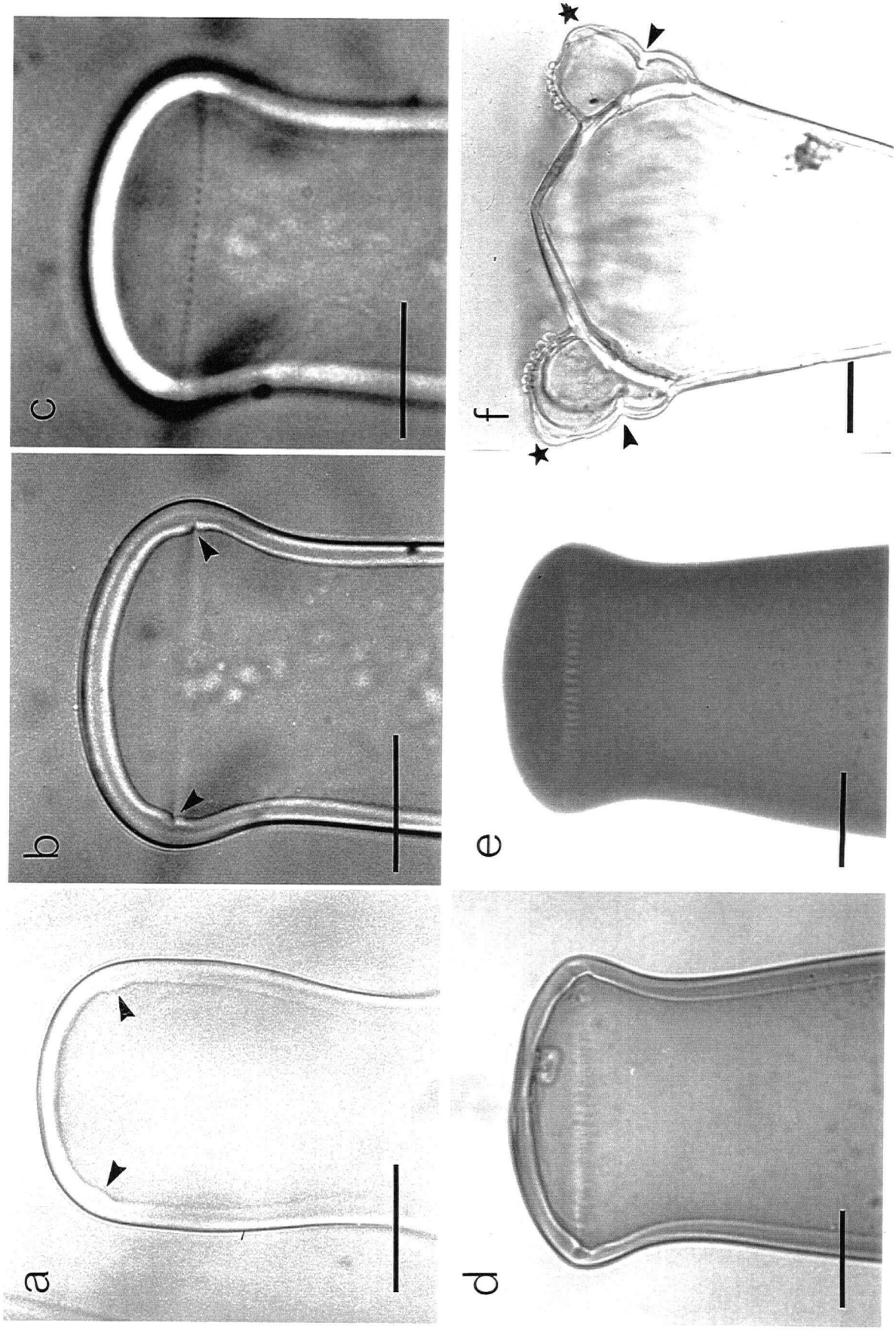


Figure 12

2.2 Whorl formation in other genera

The morphogenesis of vegetative whorls is essentially the same in all genera and the description provided for *Acetabularia* is to be used as a model. The elaboration of reproductive structures shows a greater variability between genera, and thus deserves more attention.

Polyphysa peniculus

The morphogenesis of the reproductive whorl comprises the same stages as *A. acetabulum* but the lysis spacing is larger in *Polyphysa peniculus* (43 μm for *P. peniculus* compared to 5 μm for *A acetabulum*). As a consequence, the cap of *P. peniculus* has fewer appendages (10-15 instead of 70-80) and these remain separated. The growth of the cap's appendages is somewhat intermediate between the vegetative whorl and the reproductive whorl of *Acetabularia* (Figure 13a). Among the characteristics responsible for that intermediate state are the absence of radial projections from the coronae inferior and superior and the whorled disposition of the corona protuberances (Figure 14a). The difference between the two genera arises possibly from the difference in spacing. Because of the close lateral packing in *Acetabularia* (small spacing), tangential growth is very limited compared to radial growth. As a result the fully grown cap shows radially elongated coronal chambers with the coronal protuberances arranged along a line and two radial projections from the coronae inferior and superior. Without this spatial constraint, the cap of *Polyphysa* develops more like the vegetative whorl where the packing is minimal. The presence or absence of a radial projection from the corona inferior is used as the major character to define the two genera (Berger and Kaever, 1992). Interestingly, at the same time this criterion separates the species in non-overlapping groups with respect to the number of appendages in the cap (Figure 14b). The greater number of cap appendages in all species of *Acetabularia* has two possible explanations: first, the spacing might be smaller as suggested by the measurements made on *P. peniculus* and *A. acetabulum*; second, the spacing might be similar but the tip larger in species of *Acetabularia*. Either way, the lateral packing will be greater for *Acetabularia* and could therefore explain the

difference between the two genera (Figure 14c). Further evidence that packing is in part responsible for the cap morphology of *Acetabularia* is given by *A. caliculus*. Because it is located at the lower end of the spectrum for *Acetabularia* (Figure 14b), the morphology of the corona superior comes closer to what is seen in *Polyphysa*, i.e., the segment of the corona superior shows little radial elongation and the protuberances are in a whorl pattern whenever more then two are present. The trend is not as clear in other species of *Acetabularia* located with similar low number of appendages (e.g., *A. farlowii* and *A. dentata*) so that further investigation of the possible continuous transition between the two genera would be necessary. The last evidence for the effect of close packing on morphology is given by *A. acetabulum* itself. Even if the species contains the greatest number of appendages in the cap it has been observed that the corona protuberances first appear as a whorl and only ulteriorly are they stretched along a radial line (Howe, 1901; Valet, 1968). Figure 12f shows also that early in development the coronal chamber of *A. acetabulum* is not different in any major way from the coronal chamber of *Polyphysa*. The difference in adult morphology will arise when further radial growth of the coronal chamber will have forced two radial projections along the upper and lower sides of the gametophore (Figure 14a).

Batophora oerstedi

Batophora differs substantially from *Acetabularia* and *Polyphysa* . First the hair whorls are not shed regularly as for *Acetabularia*. Therefore, at any time, the stalk of *Batophora* will show almost all the whorls produced throughout the life cycle and not simply the few most recent ones. Second, the gametophores are not formed as part of a specialized terminal structure like the cap of *Acetabularia* and *Polyphysa*; they arise instead during a second "differentiation wave" on what appeared first to be vegetative whorls (Figure 13b). This secondary wave can lag several weeks behind the actual differentiation of the thallus during which the vegetative whorls are produced. In the process, segments of first, second and third order differentiate one to three subapical spherical gametophores. Compared to *Acetabularia* and *Polyphysa*, the morphology of the reproductive whorl of *Batophora* is one step closer to the morphology of the vegetative whorl.

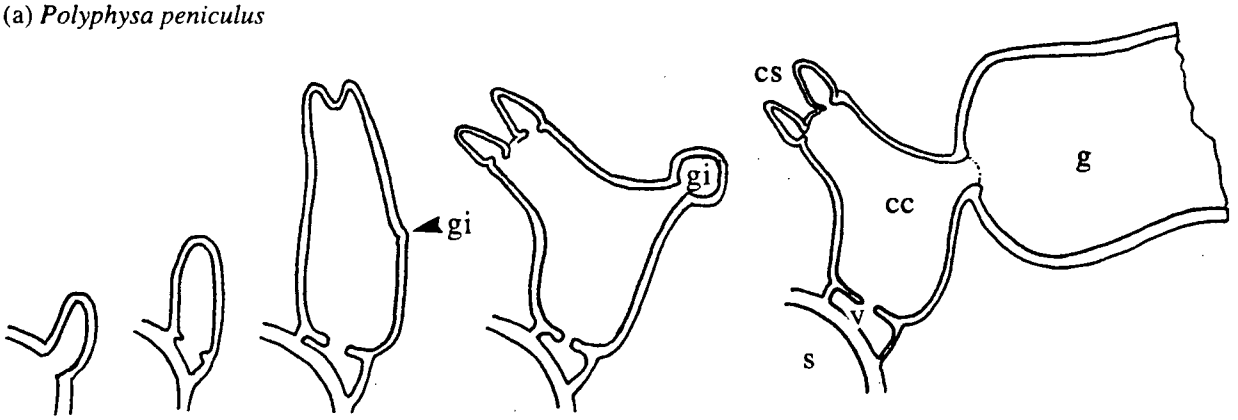
Neomeris dumetosa

The account given here is based on Church's thorough description of the development of *Neomeris dumetosa* (Church, 1895). He divided the differentiation of the thallus into five stages. The first four stages are complex variations of the vegetative whorl and they will not be discussed here save only to say that, like *Batophora*, the vegetative whorls remain attached to the stalk and will even form a dense cortex around it. The gametophores are differentiated last on structures identical to the vegetative whorls (Figure 15a). They are spherical outgrowths connecting, via a short pedicel and a perforated septum, the branching point of primary hair segments. The lag between the two differentiation processes is here very much reduced.

Halicoryne spicata

The morphogenesis of *Halicoryne spicata* was first described in detail by Valet (1968) and the information presented in this section has been drawn from his work. The representatives of the genus, after a purely vegetative juvenile phase, produce vegetative and reproductive whorls alternately. Again the morphogenesis of the vegetative whorl doesn't appear to differ in any great degree from what has been observed for *Acetabularia*, but the elaboration of the reproductive whorl differs altogether from what we have seen previously. Valet's description starts at the stage of initial growth, i.e., he didn't consider the three early stages I described. The reproductive whorl is initiated as 7-10 more or less pointed protuberances. As they elongate, the basal segment (coronal chamber) first forms a gametophore and subsequently one or two hair initial(s) branch off to constitute the corona superior (Figure 15b). The hair initial(s) and the gametophore are then partially isolated from the coronal chamber by the formation of perforated septa. In terms of pattern formation, the sequence described so far, i.e., coronal chamber growth/differentiation of the corona superior/gametophore growth, cannot be extended to *Halicoryne* where the last two steps are inverted.

(a) *Polyphysa peniculus*



(b) *Batophora oerstedi*

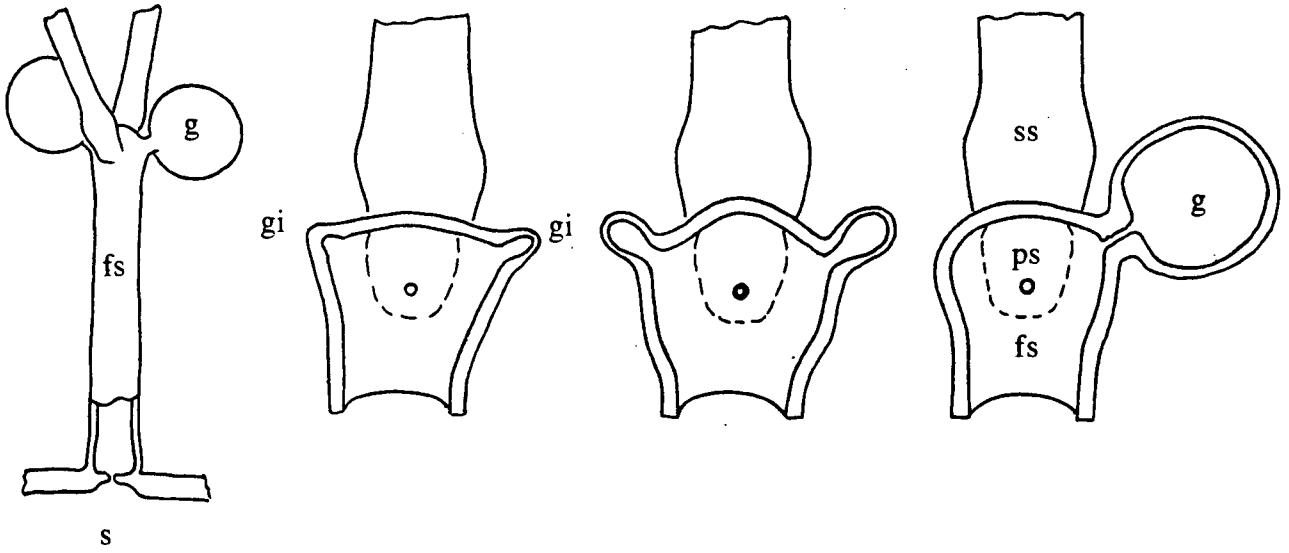
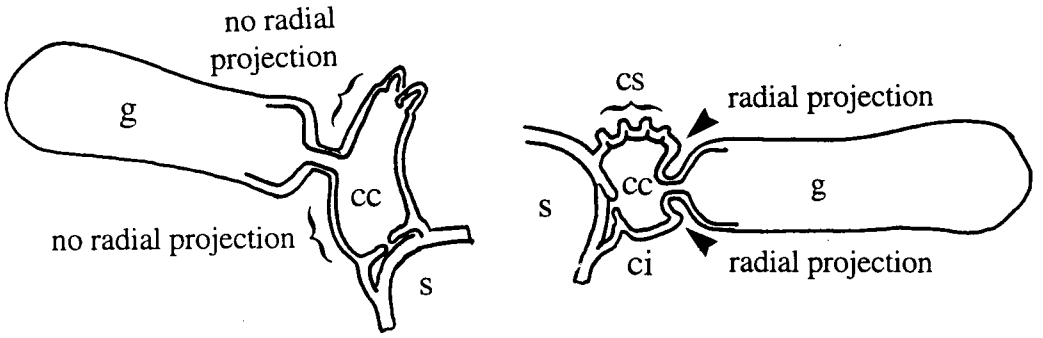
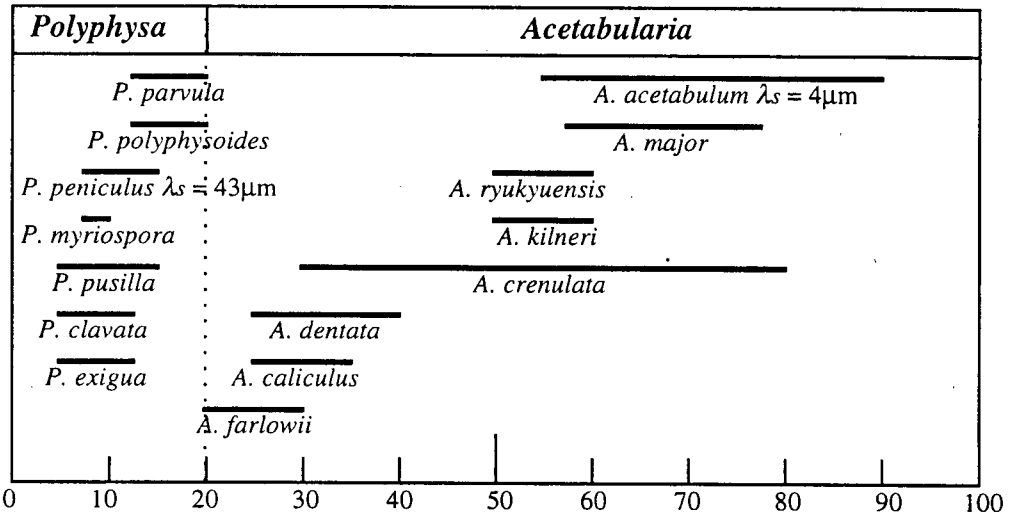


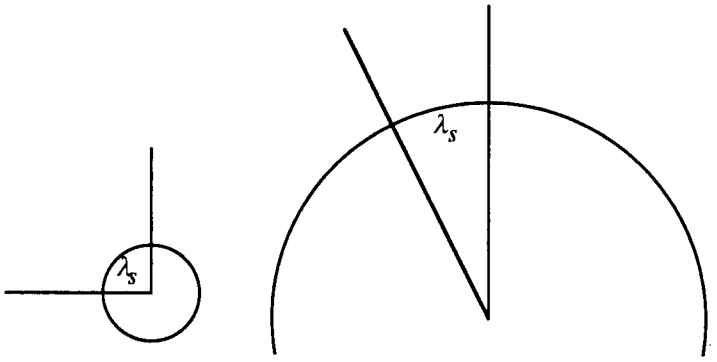
Figure 13: Morphogenesis of the reproductive whorls of *Polyphysa peniculus* and *Batophora oerstedi.* Legend- cc: coronal chamber, cs: corona superior, fs: first hair segment, g: gametophore, gi: gametophore initial, ps: preforated septum, s: stalk, ss: second hair segment, v: vestibule.



(a) *Polyphysa* *Acetabularia*



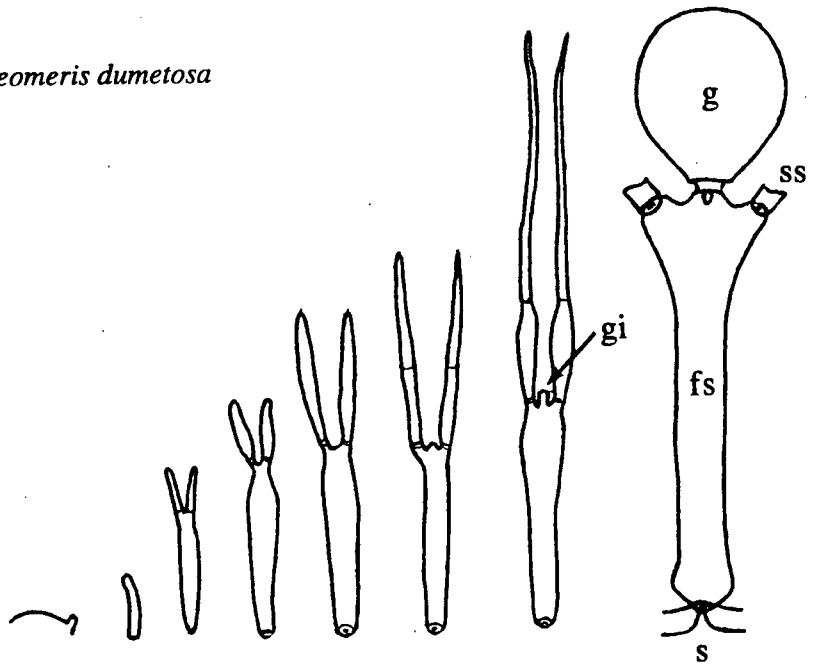
(b)



(c)

Figure 14: Qualitative and quantitative differences between the cap of *Polyphysa* and *Acetabularia*. (a) Morphology of the cap appendages. (b) Number of appendages in the cap (data from Berger and Kaever, 1992). (c) Difference in lateral interference for two sizes of cap with similar spacing. Growth tangential to the tip (circle) is very limited for a large tip. Legend- cc: coronal chamber, ci: corona inferior, cs: corona superior, g: gametophore, s: stalk.

(a) *Neomeris dumetosa*



(b) *Halicoryne spicata*

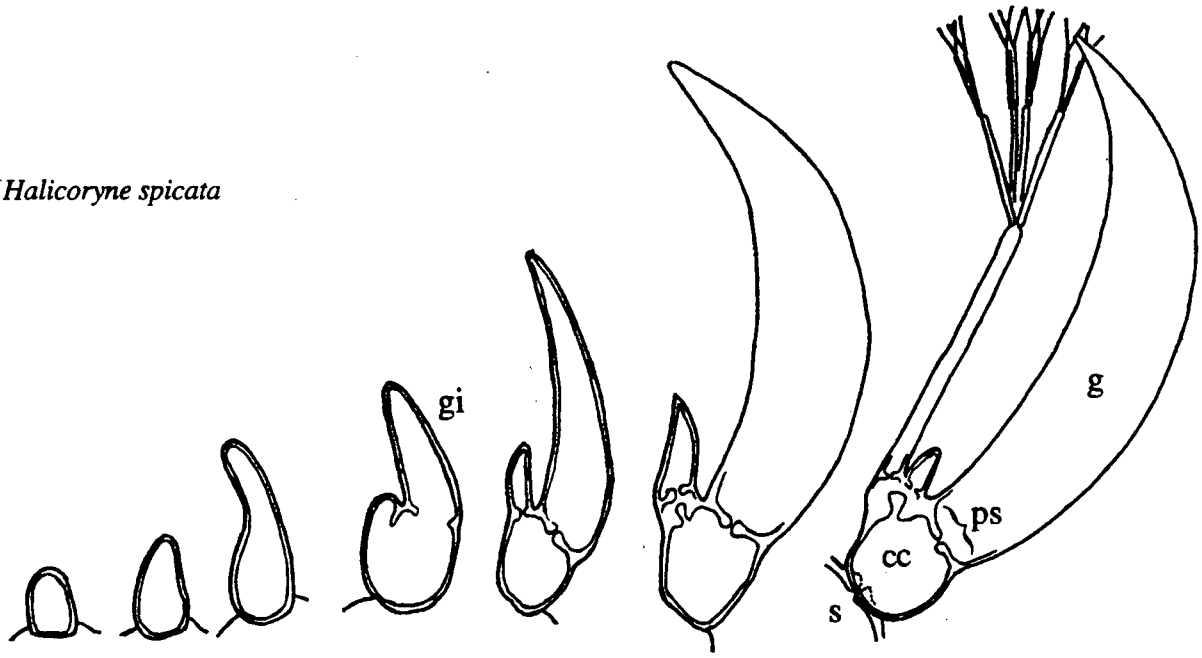


Figure 15: Morphogenetic staging of the reproductive whorls of *Neomeris dumetosa* and *Halicoryne spicata*. (a) Redrawn from Church (1895). (b) Redrawn from Valet (1968). Legend, cc: coronal chamber, fs: first hair segment, g: gametophore, gi: gametophore initial,. ps: perforated septum, s: stalk, ss: second hair segment.

2.3 Normal and teratological variation

The morphogenetic sequences given are somewhat incomplete since they don't suggest anything of the variability found within and between organisms of the same species. I would like to end this section by showing how variable the sequences really are. To go further in that direction, one needs a rule to distinguish between normal and teratological variation. I will consider "normal", forms that differ only quantitatively from the sequence exposed earlier in this section, and I will consider "teratological", forms that differ qualitatively from the sequence (i.e., if the pattern forming events are changed). In general, normal variation will not influence the organism's development in any significant way and may not even be noticed without a careful examination. A consideration of these variations will give some insight into the control and the dynamics of morphogenesis.

Normal variation

The first example that comes to mind is the variation in the number of elements within a whorl. Reports of this fact have been continuous since Nägeli (1847) and it has always been clear that this number is not under strict morphogenetic control. Only much later (i.e., Harrison *et al.*, 1981), was it shown that what is preserved from one whorl to the next is the spacing between their constitutive elements (see section 1.2). Though the spacing tends to be strictly regulated for given growth conditions, a closer look reveals that variation does occur even within a whorl. End-on observations of young whorls allows one to study such variation. The variation is usually not random but graded (from larger spacing to shorter spacing). Other examples of graded distribution (correlative variation) are numerous as shown in Figure 16. They underline the communicative nature of the morphogenetic mechanism. That is, the elements within a whorl *talk to each other.* This communication can take multiple forms (e.g., chemical diffusion, mechanical stress, etc.). When isotropic communication is achieved and sufficient time is allowed to reach a steady state in communication then the whorl is homogeneous. If communication is anisotropic or morphogenesis

too fast then the whorl might show a random or a graded distribution of spacing and development (Figure 16).

Teratological variation

While normal variation give us insights into the dynamics of whorl formation, teratological variation sheds light on its control. Two classes of events will be addressed, i.e., loss of apical dominance, and organ abortion and/or reversion. It has been pointed out that whorl formation at the tip of a lateral and whorl formation on the main axis are essentially the same (section 2.1). This statement is correct as far as whorl formation is concerned but the two processes diverge with respect to apical dominance. Laterals are devoid of apical dominance which means that whenever they branch they do not resume tip growth. The main stalk obviously has apical dominance which allows the cell to produce several whorls along a unique axis. That is to say that sustained growth (indeterminate growth) is exclusive to the main axis. Several cases of lost of apical dominance have been reported in the literature or observed in the course of this work (Figure 17). These cases have in common a redirection of sustained growth along laterals. The newly defined axises will usually produce vegetative whorls and ultimately a cap.

The absence of apical dominance in the appendages during "normal" development is further evidence for the involvement of two stages in whorl formation. The first stage, as described in section 1.3, could provide the apical dominance necessary for tip growth and the sporadic transition to an annular region before whorl initiation along the main axis. The second stage would break the continuous annulus into a whorl pattern and remain active in the appendages. Given the small size of the latter, the second stage could account alone for the subsequent branchings into 4-5 secondary segments; but, without the first stage, the appendages would be devoided of apical dominance. This could also explain why whorls on the main stalk are formed at irregular intervals while the hairs, once initiated, branch inevitably.

The second class of teratological variation, whorl abortion and/or reversion, is also instructive. The reproductive whorl is more sensitive than the vegetative whorl and therefore prone to abortion. The reversion from reproductive whorl to vegetative whorl is found frequently in *Polyphysa* and on occasion in *Acetabularia* and other genera. It is clear from these observations that the *programs* behind vegetative and reproductive whorl formation are not incompatible since vegetative organs are formed readily on structures initiated first as reproductive organs.

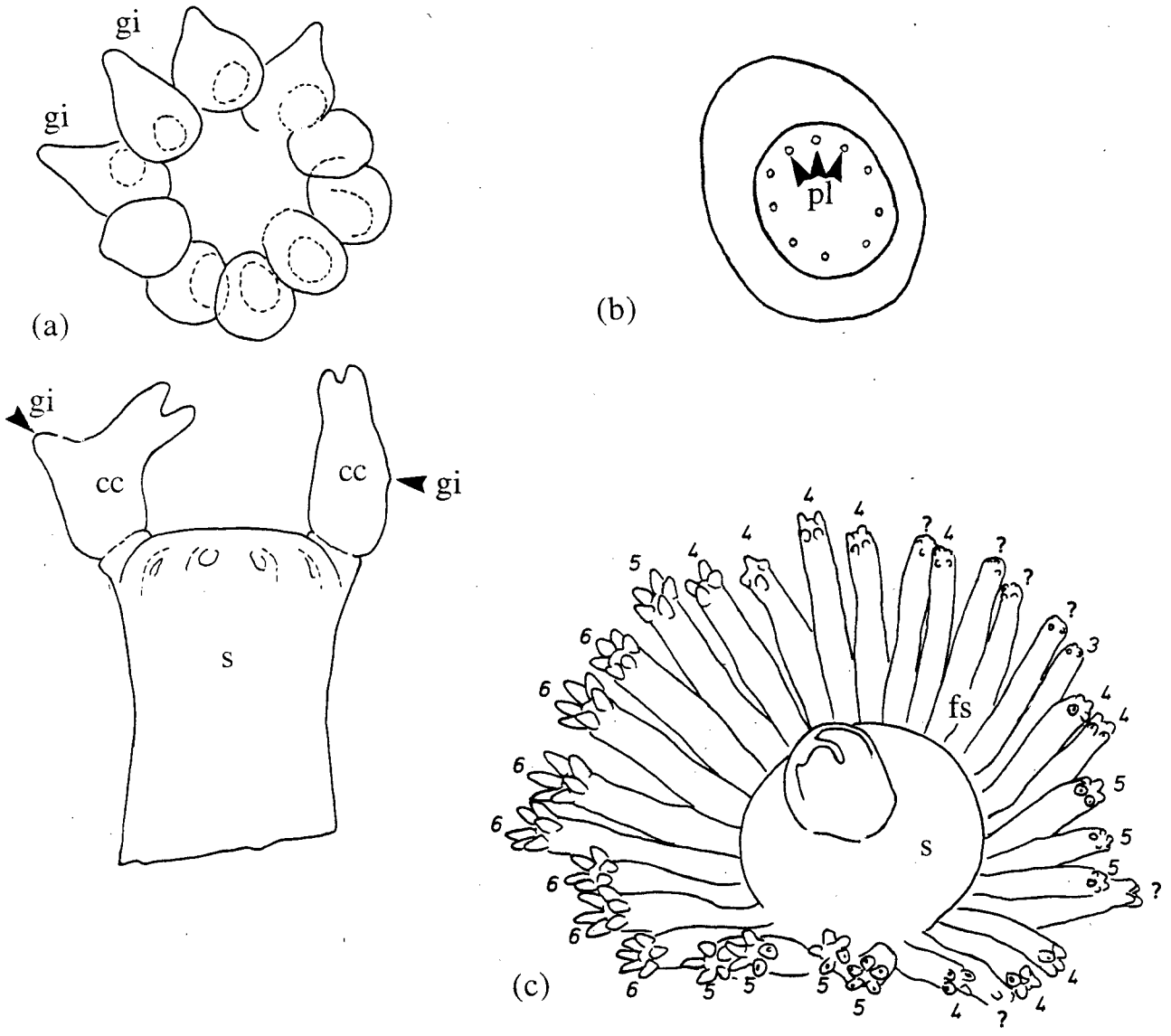


Figure 16: Normal variation of morphogenesis. (a) Cap formation in *Polyphysa peniculus*. Upper part: end-on view of the cap showing the different stages of development of the gametophores. Note how the degree of development correlates with the position in the whorl. Lower part: lateral view of the same cap showing a fairly large (left arrowhead) and the very young (right arrowhead) gametophore initial. (b) End-on view of the lysis in a vegetative whorl showing small variations in the spacing that tend to correlate with the position in the whorl. (c) Initiation of secondary hair segments. The number of inittials and their development correlates with the position in the whorl (based on a SEM of *Acetabularia acetabulum* published in Berger and Kaever, 1992). Legend- cc: coronal chamber, fs: first hair segment, g: gametophore, gametophore initial, pl: punctate lysis, s: stalk.

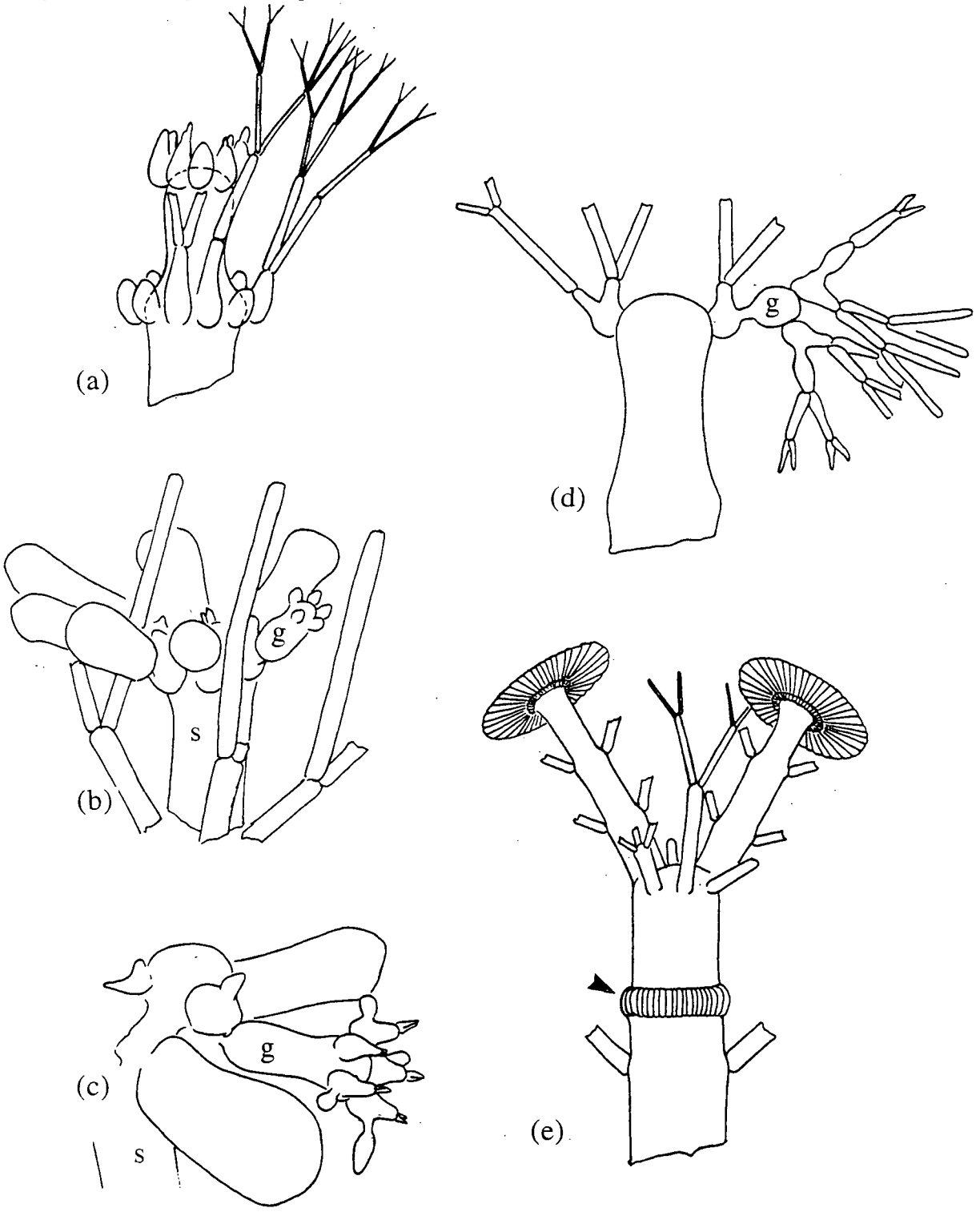


Figure 17: Teratological variation of morphogenesis. (a)-(d) *Polyphysa peniculus*, (e) *Acetabularia acetabulum*. (a) Two caps aborted. (b)-(d) Different degrees of development of small caps on a gametophore. (e) Loss of apical dominance of the stalk to two hairs. A cap was aborted earlier (arrowhead). Legend- g: gametophore, s: stalk.

Chapter 3

The evolution of morphogenesis

3.1 Fossil Sequence

As a consequence of their natural tendency to calcify, the Dasycladales algae left behind them an extensive fossil record. The first representatives appeared as early as 570 million years ago (Cambrian). To date, 200 genera have been reported representing almost 900 species (Barattolo, 1991). Compared to these numbers, the 11 extant genera (38 species) can account only for a very small fraction of the overall diversity of the group. I do not intend to cover all of that diversity but rather, I want to give a general impression of the major evolutionary trends leading to the extant species. Two such trends, *thallus elaboration* and the *distal shift of reproductive structures* are very suggestive of how morphogenesis was changed during the evolution of the Dasycladales.

Thallus elaboration

The first stage has been characterized as "an irregular stem-cell, sometimes recumbent, branching, or even anastomosing, with a dense and irregular arrangement of branches " (Herak *et al,* 1977, page 146)(Figure 18). But very early (i.e. Cambrian-Ordovician) a cylindrical main stalk became predominant within almost all taxa up to recent time. This second stage is also characterized by an *aspondyl* morphology (i.e., random distribution of laterals on the stalk) and unbranched laterals. The third stage differs from the preceding stage by its *euspondyl* (i.e., whorled) distribution of unbranched laterals. It has been reported that the aspondyl-euspondyl transition might have occured three times (Pia, 1923) or even seven times (Kamptner, 1958). Typical representatives of this stage date from the Carboniferous. The fourth and last stage is characterized by a cylindrical axis with euspondyl branching laterals. All extant species fall in this group.

Distal shift of reproductive structures

This trend can also be divided into four stages (Barattolo, 1991)(Figure 18), each new stage shifting the reproductive structures one step away from the main axis. The first stage is described as an *endosporate* type where the cysts are developed inside the central cavity. This stage is first reported from the Cambrian and reappears sporadically throughout the fossil sequence. In the early Carboniferous the second stage emerged. The latter is described as a *cladosporate* type, the cyst being formed in first order segments of laterals. The Triassic saw the appearance of a third stage. The morphology, where the cysts are contained in a specialized ampula, is refered to as *choristosporate*. This stage is typical of the extant representatives of sub-family Dasycladaceae. The gametophores, usually borne on the primary hair segment, can be terminal, subterminal or lateral. The fourth stage, the *umbrellosporate* type, is simply a remodelling of the preceding stage and is represented by the Acetabulariaceae sub-family. It is interesting to note that some teratological cases in extant species are simple reversions to stage 1 or 2 (Valet, 1968). This trend is generally accepted but the exactly opposite trend, i.e. choristoporate -> endosporate has also been held (Emberger, 1968).

Herak *et al* conclude from their study that "the degree of continuity of a particular feature during the past is proportional to its regularity and reversely proportional to the degree of its differentiation and specialization. That means that the regularity, non-differentiation and non-specialization may be considered as indications for the linking of different taxa throughout the time-space, and as a source for sporadic specializations and iterative revival of some features" (Herak *et al*, 1977, page 148).

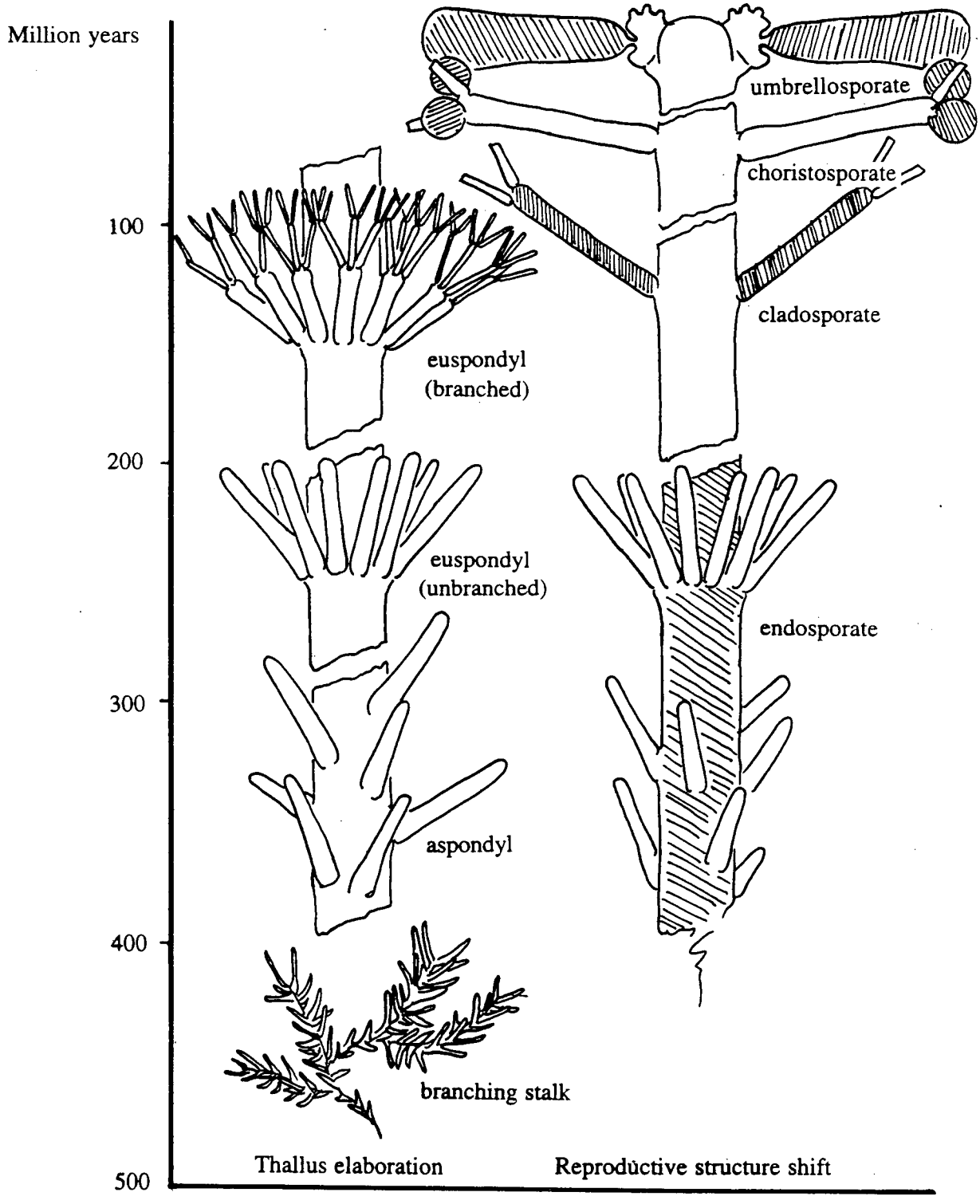


Figure 18: Two major trends of the fossil sequence. The cysts will develop in the crosshatched regions

3.2 Whorl homology

The morphogenetic similarities described in section 2.1 and 2.2 point to the deep homology between the vegetative and the reproductive whorl. This fact has been recognized since Nägeli (1847) but is now often overlooked. As a consequence, the vegetative and reproductive whorl are often treated as structures foreign to each other and few comparative studies have been done. A review of the homological systems proposed may thus turn out to be useful. To my knowledge these models have been reviewed only once as part of Diedrik Menzel's Ph.D. thesis (1982). I will focus mainly on *Acetabularia* since in that genus the two whorl types are most different. This discussion will lead to what I think is the most likely homological correspondence between the different parts of the vegetative and reproductive whorls. This information will then be used to find what is needed morphogenetically to go from the vegetative whorl to the reproductive whorl.

We owe the first homological system to Nägeli (1847). It was considered by Church as the "older view", a view he still believed in (Church, 1895)(Figure 19a). As he described it, "the formation of a cap-whorl in *A. mediterranea* (i.e., *A. acetabulum*) seems to be accompanied by a telescoping of the main axis in the neighbourhood of the cap, and the superior and inferior coronae may be regarded as belonging to the main axis rather than to the cap-rays. The marked radial arrangement of the scars on the corona superior of *A. mediterranea* seems to point definitely to such a correlation" (Church, 1895, p. 595-96). Following this idea, the gametophore would be the first segment of a modified hair and the protuberances of the corona superior an aggregate of whorls brought together by an extreme reduction of the interwhorl segments (telescoping). Falkenberg (1882) had put forward a similar idea as that expressed later by Church but he had suggested also that the early abortion of one whorl would lead to the corona inferior (Figure 19a). This proposal is not without similarities with the current theory about the origin of the angiosperm flower where the perianth, the corolla, the androecium, and the gynoecium are thought to be complex aggregates of modified leaves.

One year before Church's paper, Solms-Laubach (1894) had presented a new proposal based on his observation of a perforated septum separating the central cavity and the coronal chamber (Figure 19b). This discovery threw the older view into suspicion as it became difficult to argue for a natural transition (telescoping) between the central cavity and the coronal chamber. Consequently Solms-Laubach was led to reinterpret the coronal chamber and the protuberances of the corona superior as homologous to the primary and the secondary hair segments respectively. He held also a different view about the origin of the gametophore. For him, it had to be compared with the gametophore of *Bornetella* (in that genus the gametophore(s) is formed laterally on the primary hair segments). Solms-Laubach thus assumed that the gametophore in *Acetabularia* could only be homologous to another gametophore, implying that the organ should stand on its own as a *sui generis* organ. Howe (1901) held a similar view but added that the lateral projections forming part of the coronae superior and inferior were also to be recognized as modified gametophores (Figure 19b).

More recently, Valet (1968) put forward a third proposal, where the corona superior, the corona inferior as well as the gametophore are homologous to secondary hair segments (Figure 19c). In this case the protuberances of the corona superior would be equivalent to third order hair segments. This proposal is part of the tradition in interpreting the gametophore as a modified hair.

The detailed staging of section 2.1. leaves little doubt about the correspondence that has to be drawn between the coronal chamber and the primary hair segment. The shared morphogenetic stages (Stage 1 to Stage 4) point toward the deep homology between these structures though the homology tends to be slightly obliterated as they undergo diverging growth processes. Given this basis, Church's homological system (the "older view") can easily be ruled out. Two questions remain to assess the worth of Solms-Laubach's and Valet's systems: i) what is the nature of the lateral projection typical of the coronae superior and inferior? and ii) is the gametophore (Stage 5) a modified hair or a *sui generis* organ?

In relation to the first question, it has been mentioned that Falkenberg (1882), Howe (1901) and Valet (1968) regarded the two coronal projections and the gametophore they flank as three equivalent structures, the former two being simply reduced or aborted in course of development while the latter would grow into the typical club shaped reproductive organ. This idea is hardly tenable once one realizes that the coronal projections don't show the typical "localized" initiation like as the punctate lysis at the origin of the hair (Figure 11a) or the local bulge at the origin of the gametophore (Figure 12b,c). This localized initiation has its final expression in the more or less pronounced pinching at the point of origin of *bona fide* appendages (hair and gametophore), a feature conspicuously missing for the coronal projections. Figure 12f provides futher evidence against this hypothesis in showing that at the time the rays are initiated as small lateral bulges, the coronae inferior and superior show no similar bulging. I would therefore prefer to explain the origin of the coronal projections not in terms of pattern formation but in terms of growth (see the discussion in section 2.2).

As for the second question, a deeper enquiry into the origin of the gametophore would probably allow one to discriminate between these contradictory proposals. The original claim made by Solms-Laubach was based mainly on the location of the gametophore. For him, the gametophore had a dual origin; when it grew in the vicinity of secondary hair segments, as for *Batophora* or *Dasycladus*, it had to be considered as deriving from one of them; when it grew at some distance from the branching point, as for *Bornetella*, it had to stand on its own as a *sui generis* organ. It's the tight parallel he was able to draw between the structure of *Bornetella* and *Acetabularia* that led Solms-Laubach to the homological proposal given. This idea is in line with the long known morphological rule " that the most conservative criterion of structural homology is relative position and connections" (Boyden, 1973, page 92). Pia (1920), who also granted the gametophore of *Acetabularia* an independent origin, based his view on a different criterion, i.e., the lag between the initiation of the adjacent hairs and the gametophore itself. This fact was underlined in section 2 where, with the exception of *Halicoryne*, it was shown that gametophore initiation and growth is

the last stage in the morphogenesis of a reproductive whorl. This culminating stage lags from a few hours (*Acetabularia*) up to several weeks (*Batophora*) behind the preceding stage. Therefore, in Pia's mind, no matter how close a gametophore could come to occupy the place of a hair in a given whorl, the fact that it didn't develop concomitantly with the neighbouring hairs was enough to credit it with an independent evolutionary origin.

What is implied in Pia's "temporal argument" or Solms-Laubach's "spatial argument" is strengthened when conjointly reinterpreted within the pattern formation viewpoint. Currently, several biological observations strongly suggest that the whorl pattern in Dasycladales ought to be considered as an "entity" in its own right (see Harrison, 1993 for a discussion of pattern as entity). Among the points addressed in this work are:

1) The initiation of the elements of a whorl is simultaneous (section 2.1).

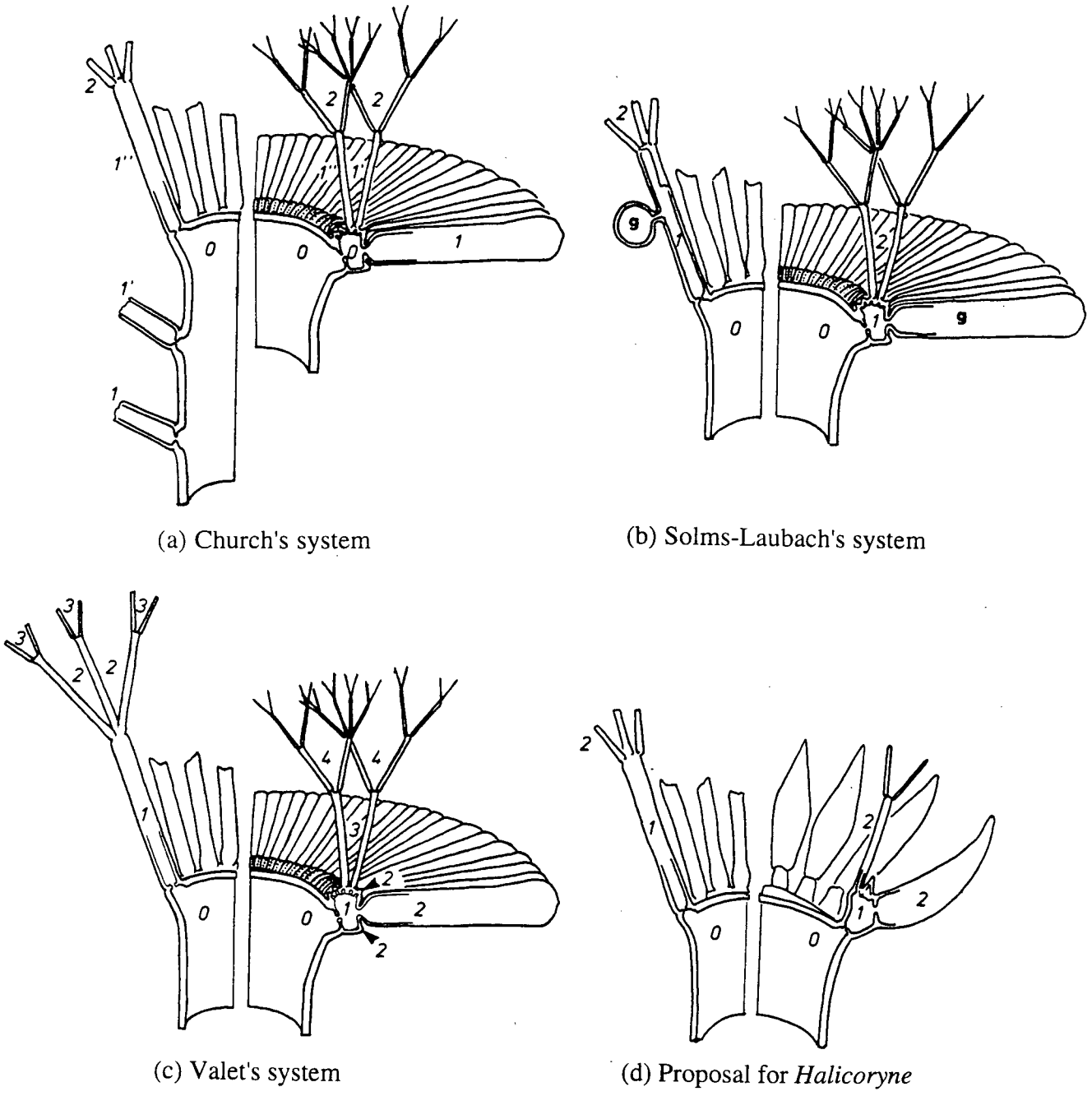
2) The initiation is made along an extremely precise band (almost a line) circling the tip (section 2.1)

3) The whorl is internally coherent because of communication controlling the spacing and the development of the whorl elements (section 2.3.).

It is significant that all the models proposed so far show exactly these types of behaviours.

If these three observations are sufficient to consider the whorl as an entity then one cannot hold the idea that, in the course of evolution, one of the whorl elements has been displaced in time and in space to serve a reproductive function. The integrity of a whorl is preserved only if the gametophore is considered as an evolutionary innovation, an additional morphogenetic stage, a *sui generis* organ. Therefore one additional stage must be added to the two stages needed for whorl formation in order to account for the growth of the gametophore in the reproductive whorl (see section 3.3).

At this point we must go back to the exceptional case of *Halicoryne*. The staging presented in Figure 15b already suggests the uniqueness of *Halicoryne*'s morphogenesis since the gametophore appears very early in the sequence while it is the concluding stage in other genera. Moreover, the gametophore is closed by a perforated septum, a feature not seen anywhere else among the Acetabulariaceae where *Halicoryne* is usually classified but observed by Church (1895) for *Neomeris*. In this case the gametophore might be a modified hair (Figure 19d; Emberger, 1968) but a closer examination would be necessary to assert its origin.



(a) Church's system

(b) Solms-Laubach's system

(c) Valet's system

(d) Proposal for *Halicoryne*

Figure 19: Homological systems. (a) Church's homological system (1895). Following Falkenberg (1882) the coronal projections are homologous with first hair segments. (b) Solms-Laubach's homological system (1894). Following Howe (1901) the coronal projections are aborted gametophores. (c) Valet's homological system.(d) Proposal for *Halicoryne* also described earlier by Emberger (1968).

3.3 Major innovations in pattern formation

The acceptance of Solms-Laubach's homological system and the new proposal for *Halicoryne* bring a new light on the evolution of Dasycladales as a whole (Figure 20). The ancestral stock was probably an euspondyl-cladosporate form. The elaboration of the thallus had already reached its end point at that time but the distal shift of the reproductive structures was still at an early stage (i.e., endosporate or cladosporate). Two new lines were formed depending on how the requirement for a gametophore imposed by the latter trend were resolved. One line, today represented by *Halicoryne*, evolved a gametophore by specializing a vegetative hair. The morphogenesis of *Halicoryne* still shows this origin. A second line was formed where gametophores were neo-formations and it now accounts for most Dasycladales genera. The second line underwent an additional branching leading to the well established subfamilies Acetabulariaceae and Dasycladaceae. Their common ancestor could be thought to have been a *Batophora*-type alga (Valet, 1968). The two new lines followed different trends. The Acetabulariaceae reduced the number of reproductive whorls to one or a few. The "coronal chamber" and the gametophore underwent a deep remodelling, the higher order segments (i.e., the hairs growing from the corona superior) remaining unchanged. The Dasycladaceae line followed a different trend where the primary segments and the gametophores are most of the time unmodified while the second order segments are strongly modified.

This evolutionary scheme, based on morphogenesis, is slightly different from those attained with different approaches. The distinction between Acetabulariaceae and Dasycladaceae seems fairly constant for all the approaches, whether they are based on adult morphology (Berger and Kaever, 1992), the fossil record (Pia, 1920) or on ribosomal DNA sequence (Olson *et al*, 1994). Menzel (1982) is to my knowledge the only other researcher who contrasted *Halicoryne* with the rest of the Acetabulariaceae. He justified the distinction on the basis of the presence of a perforated septum between the coronal chamber and the gametophore.

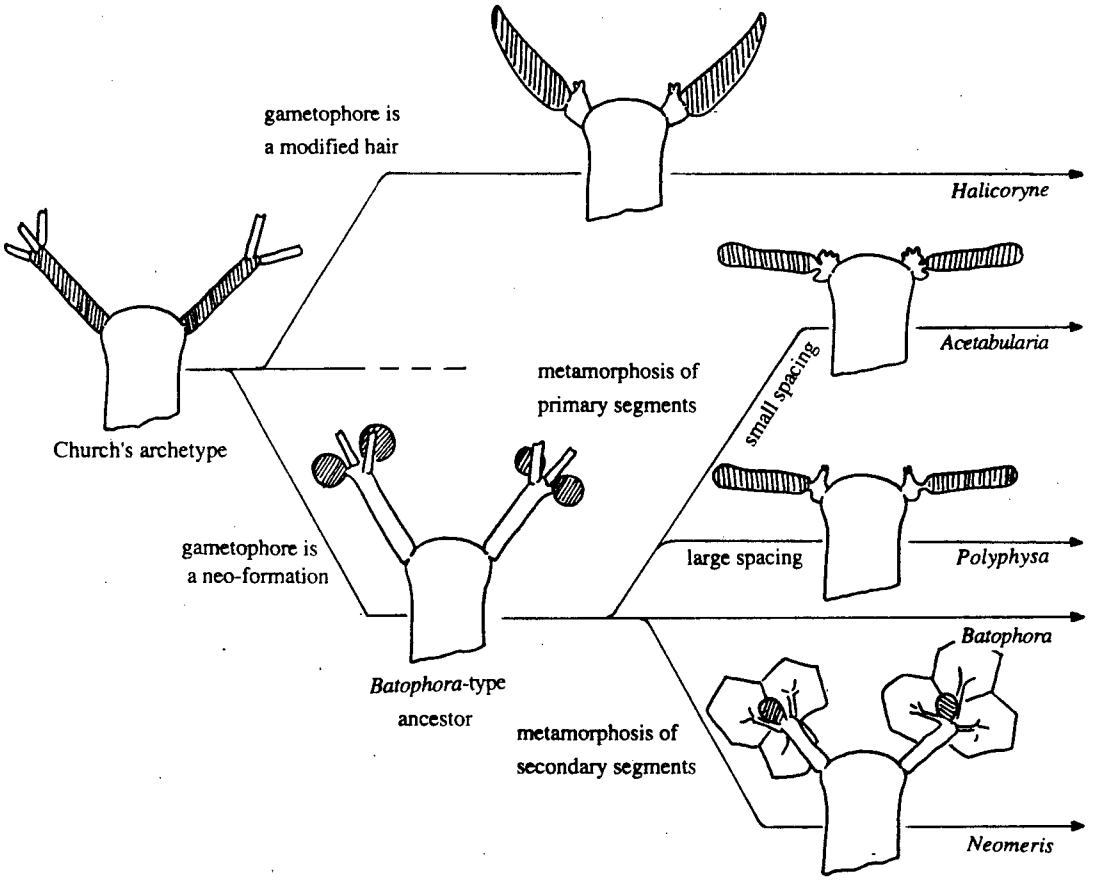


Figure 20: Major innovations in pattern formation. The cysts will develop in the crosshatched regions.

Chapter 4

Distribution of free and bound calcium during morphogenesis

4.1 Introduction

With the recent development of highly specific fluorescent dyes for Ca^{2+} the knowledge about this important ion has increased tremendously (for a review see Tsien, 1994). It is now clearly established that intracellular Ca^{2+} concentration and the transport of Ca^{2+} across the cell membrane is critical for tip growth. The multiple roles of Ca^{2+} in tip growth have been exposed in section 1.3. The key role of Ca^{2+} is sufficient to motivate further research in the Dasycladales. Additionally, the models presented involve directly or indirectly Ca^{2+} ions in their dynamics so that a close look at Ca^{2+} ions might also reveal to what extent the assumptions of the models are correct.

Reiss and Herth (1979) provided the first description of Ca^{2+} distribution in *Acetabularia* using chlorotetracycline (CTC). They found a high Ca^{2+} concentration at the growing tip of the stalk and hairs, and at the base of each hair. Because the affinity of CTC with divalent cations is greater in non-polar solvent, CTC reveals mainly membrane-bound Ca^{2+} (Caswell, 1979) (Reiss and Herth reported also an important fluorescence in the cell wall). These results were reproduced by Cotton and Vanden Driessche (1987) using CTC and aequorine. The latter targets specifically free cytosolic Ca^{2+}. It was also observed that the apical gradient of Ca^{2+} disappeared as soon as the cap was initiated.

More recently, measurements with Ca^{2+}-selective microelectrodes gave a cytosolic free Ca^{2+} concentration of 560 nM (Amtmann *et al*, 1992). Removal and re-addition of 10 mM external Ca^{2+} induced corresponding changes of about 50 nM of the cytosolic Ca^{2+}. A kinetic analysis of the cytosolic Ca^{2+} response to such removal of external Ca^{2+} suggested a powerful transport system.

The latter is necessary to keep the low cytosolic free [Ca^{2+}] despite the very high Ca^{2+} levels in the environment (7 mM).

The distribution, tight regulation and low concentration of Ca^{2+} suggest a possible role as a second messenger (see Vanden Driessche, 1990 for additional evidence). The parallel distribution of calmodulin (Haußer *et al*, 1984; Cotton and Vanden Driessche, 1987) suggests that the ion and the protein could act together in the signal transduction pathways.

Despite its importance, a major shortcoming of this earlier work is that it is centered mainly on fully grown whorls. Similar work need to be undertaken at critical stages of whorl formation, mainly when the whorl prepattern is layed down (stages 2 and 3, Figure 10). Such work has been done with CTC (Harrison *et al*, 1988). The detailed observations showed a redistribution of membrane-bound Ca^{2+} from a terminal maximum to a subapical annulus as the tip broadens to initiate a whorl. Shortly after initiation, the annulus is still visible at the base of the whorl, each initial showing an additional high level of Ca^{2+}. Results for the reproductive whorl and free cytosolic Ca^{2+} were still missing and therefore represent the first motivation for this work. The second motivation is to test the model's assumptions about the role of Ca^{2+} in morphogenesis.

The buckling model by Martynov doesn't make any explicit assumptions on Ca^{2+} behaviour, yet Ca^{2+} ions are known to be involved in the cross-linking of the cell wall components thus increasing its stiffness (Steer and Steer, 1989). One must expect that an increase in extracellular Ca^{2+}, by increasing the wall stiffness, would increase the buckling wavelength, i.e., increase the spacing between the elements of the whorl. This is contrary to the observed decrease in λ_s with increasing $[Ca^{2+}]_e$ (Harrison and Hillier, 1985). Despite the counter-evidence for the direct involvement of Ca^{2+} in pattern formation by buckling, the ion could be responsable for the transduction of the buckling "prepattern" into growth (Trewavas and Knight, 1994).

The dependency of the spacing λ_s on $[Ca^{2+}]_e$ suggests, when interpreted in the reaction-diffusion paradigm (i.e., Harrison's model), an involvement of Ca^{2+} as a precursor to one of the morphogens. In theory, this role as a precursor imposes little constraint on Ca^{2+} distribution. Pattern formation can occur with a uniform concentration of precursor or different gradients of precursor since reaction-diffusion models like the Brusselator appear to be stable to such variations (Holloway and Harrison, 1995). In applying the theory though, there is a need for a two stage model to account for whorl formation on a hemispherical tip, suggesting that Ca^{2+} ions are part of the first patterning event leading to a subapical annulus of high precursor concentration. The second stage would feed on this precursor annulus to eventually form the whorl prepattern. It would therefore be natural to expect a high terminal Ca^{2+} concentration during apical growth changed into a subapical annulus of high Ca^{2+} concentration whenever a whorl is initiated.

Lastly, Ca^{2+} ions figure as morphogens in Goodwin's model and they would therefore have to form a whorl prepattern prior to the initiation of the hairs. This raises the question of the role of Ca^{2+} in morphogenesis; is the ion involved as a precursor for pattern formation, as a morphogen, as a second messenger for the transduction of the prepattern into growth, or as a combination of these?

4.2 Materials and Methods

Culture

The stock culture of *Acetabularia acetabulum* (L) Silva and *Polyphysa peniculus* Agardh have been maintained in artificial sea water (Shephard, 1970) at 20 °C and a 12:12 h light/dark cycle (the light intensity ranged from 32 to 54 lx). Cells at different stages of development were usually selected during the first half of the light cycle and then stained appropriately.

CTC and Fluo-3 staining

The protocol followed for the CTC staining has been fully described by Harrison *et al.* (1988). Initial attempts of ester loading using Fluo-3 AM (Molecular Probes, Eugene, Oregon) failed. Even cells injected with the ester form of the dye showed little or no fluorescence, suggesting that plant esterase doesn't succesfully cleave the ester bond to activate the dye. Despite numerous technical problems of its own, pressure injection of the acid form of Fluo-3 has proven most succesful. The pressure was generated with a 2 ml Mechrolab (B-D) glass syringe connected to a micropipette holder through a flexible tubing (B-D) filled with mineral oil. A micromanipulator controlled the movement of the micropipette. The pressure injector was set-up on a dissecting microscope and most of the work was done at a 80x magnification. The micropipettes were pulled on a Flaming/Brown micropipette puller (Sutter Instrument Co., Model P-87) such as to form a very sharp point (5 to 10 μm) on an overall blunt tip (Figure 21a). Once glued on a micropipette holder, the micropipette tip was slightly broken by lowering it to a flat surface and then filled with mineral oil. A stock solution of Fluo-3/K in distilled water (2-10 mM) was kept frozen. When needed, 10-50 nl of the thawed solution were back filled in the tip of the micropipette. The cells were put in a thin film of growth medium before being injected at a distance of 300 to 600 μm from the region of interest (Figure 21b). After a recovery period of 5-15 minutes, the dye was visualized under a Zeiss epifluorescence microscope equiped with a fluorescein filter set. Photographs were taken on 1600 ASA Fuji Super HG colour negative film at exposures usually of 45 seconds. Prints were produced by computer scanning the negatives directly (Adobe Photoshop™, version 3.0) as all photographic processes attempted produced poor results. Controls were done following the same protocol but replacing Fluo-3 by its fluorochrome, fluorescein.

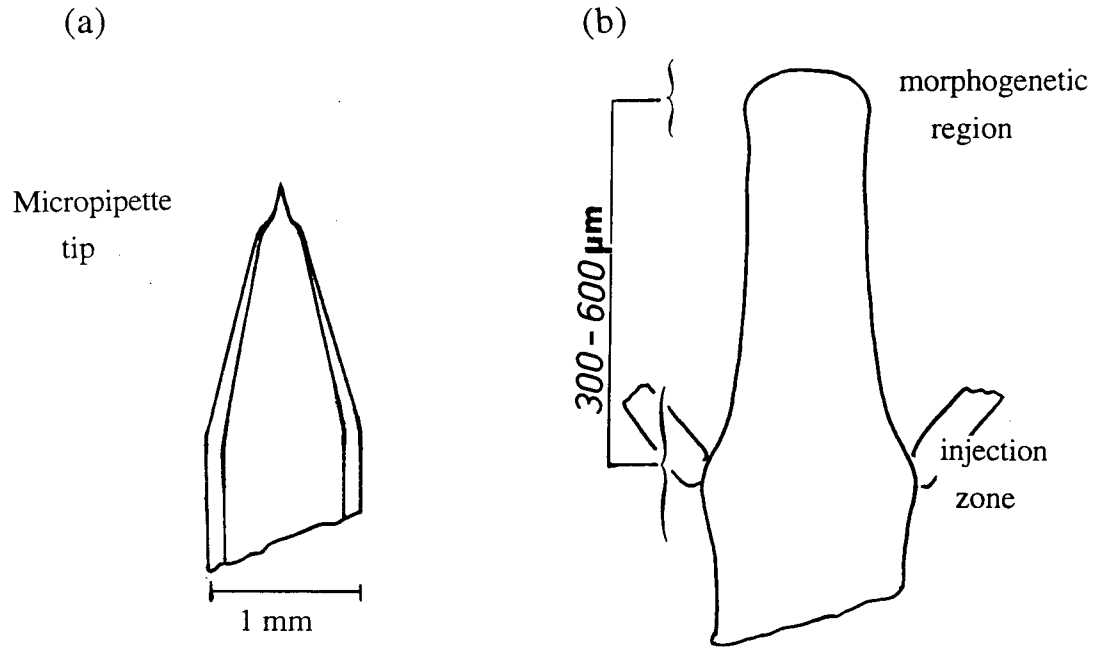


Figure 21: Micropipette and injection point. (a) Outline of the tip of the micropipette used. (b) Location of the injection zone with respect to the morphogenetic region.

4.3 Results

The results obtained with CTC show that active growth is correlated with higher concentration of membrane bound Ca^{2+}. This is compatible with previous findings for the vegetative whorl of *Acetabularia* (Figure 22a; Reiss and Herth, 1979; Harrison *et al*, 1988) and other tip growing cells such as pollen tubes (Reiss and Herth, 1978), and *Micrasterias* (Meindl, 1982). As for the vegetative whorl, the early stage of cap formation is characterized by a ring of fluorescence circling the tip. This region, initially continuous, breaks down into a series of bright spots soon after the appearance of the cap initials (Figure 22b,c). An end-on view of a young cap stained with CTC shows bright corona protuberances. The fluorescence is conspicuously greater in protuberances

actively growing into hairs (Figure 22d). Additional fluorescence can be seen in the cell wall between the cap rays. Finally, the growth of the gametophore is also associated with a high concentration of membrane bound Ca^{2+} as demonstrated in *Polyphysa* (Figure 23d,e). Overall, the results show that growth and Ca^{2+}-CTC fluorescence are in lock-step but there is as yet no evidence that Ca^{2+} ions might prefigure morphological differentiation. Morphological differentiation precedes appearance of a whorl pattern in Ca^{2+} distribution.

The staining of free Ca^{2+} with Fluo-3 provided similar results. A flattened tip initiating a hair whorl is characterized first by an annulus of fluorescence (Figure 23a,b). The annulus eventually breaks down into a whorl pattern soon after the appearance of the initials (Figure 23c). Similarly, a cap forming tip where the initials have just appeared show a continuous region of fluorescence circling the tip (Figure 24a,b). In both cases the fluorescence defines a region contrasting sharply with the rest of the stalk. Only in later stages will this continuous region break into individual spots corresponding to the growing initials (Figure 24c,d). The fluorescein controls fail to show a similar gradient of fluorescence even for well developed initials (Figure 25). A sequential staining of the same cell, first with Fluo-3 and then with CTC is also instructive. While the CTC/Ca^{2+} fluorescence is already indicative of the location of each initial, the Fluo-3/Ca^{2+} fluorescence is still continuous (compare Figure 22b and Figure 24a,b). This would suggest that the membrane bound Ca^{2+} is more closely tied to growth, though the difference in fluorescence intensity, in the size of the Ca^{2+} pool targeted, and in the optical path length (i.e., the distance covered by the incident and emitted light) of the two dyes might be enough to explain the observed discrepancy. We must conclude that, as yet, neither a bound Ca^{2+} prepattern no a free Ca^{2+} prepattern have been observed, thus morphological differentiation appears to be always a step ahead of Ca^{2+} distribution.

Figure 22: CTC staining. The red color comes from the natural autofluorescence of the chloroplasts. The yellow/orange fluorescence is produced by the Ca^{2+}-CTC complex. (a)-(d) *Acetabularia acetabulum.* (a) Flattened tip before initiation of a vegetative whorl. The tip shows an annulus of strong Ca2+-CTC fluorescence. (The annulus is seen more easily during the microscopic obseervation, on the photographs presented the annulus is suggested by the two lateral bright spots) (photograph previously published in Harrison *et al.*, 1988)(bar=75μm). (b) Early initiation of cap initials. The Ca^{2+}-CTC fluorescence already suggests their presence (bar=75μm). (c) Later stage in the development of the cap. The intense fluorescence in the actively growing cap contrast with the remaining part of the stalk (bar=75μm). (d) End-on view of a young cap. Outer ring: young cap rays (gametophores). The radial streaks of yellow/green fluorescence come from the dye trapped in the cell wall and is not of interest here. Inner ring of dots: fluorescence of the corona protuberances. Six actively growing protuberances show brighter fluorescence (bar=75μm).

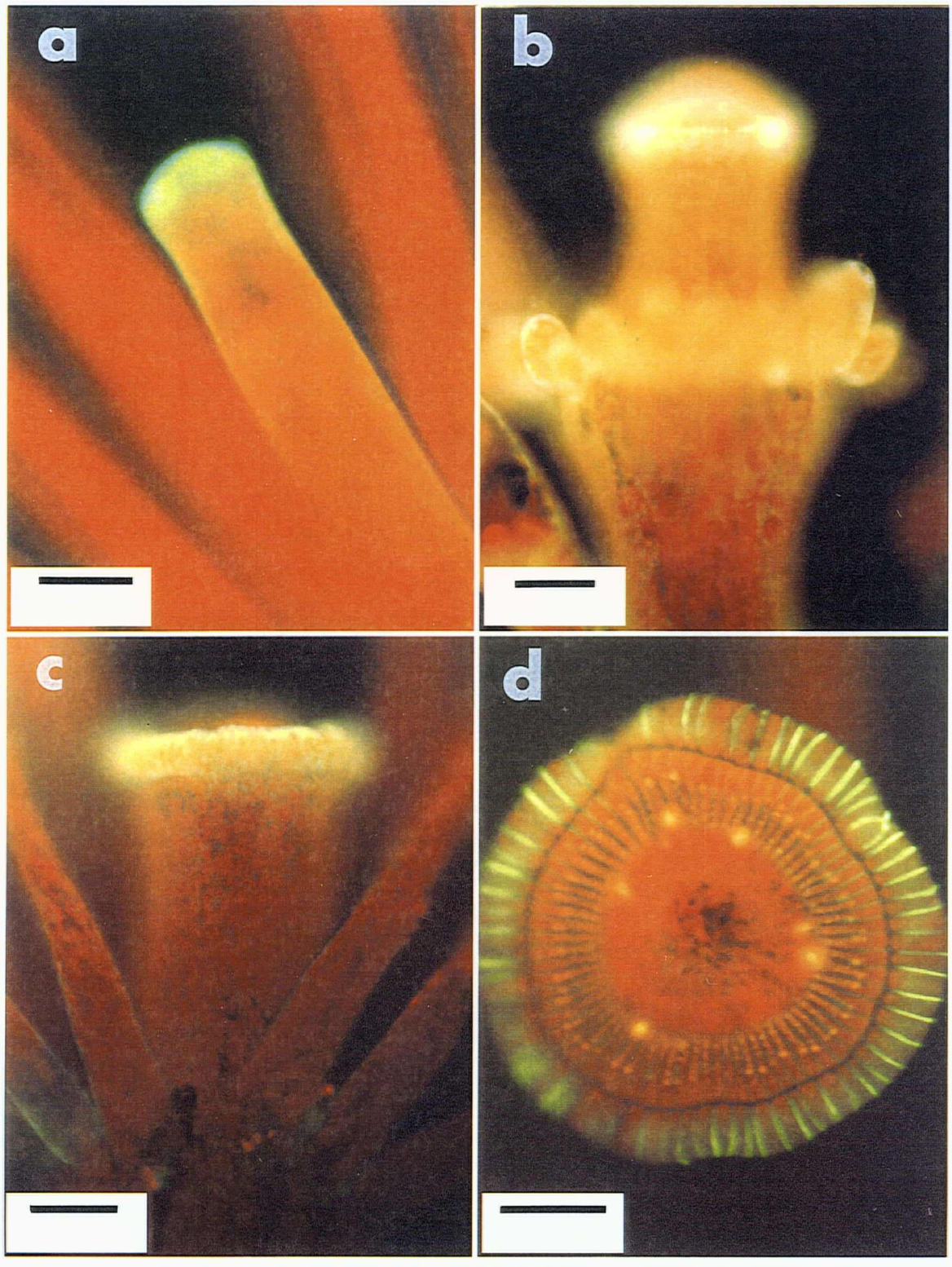


Figure 22

Figure 23: CTC and Fluo-3 staining. (a)-(b) Side growth before the initiation of a vegetative whorl in *Acetabularia acetabulum* (bar=75μm). (a) Bright field photograph showing the large central vacuole and the thin cytoplasmic layer in the periphery. (b) Ca^{2+}-Fluo-3 fluorescence. The tip morphology and the distribution of Ca^{2+} are strikingly similar to the CTC observation (Figure 22a). (c) Ca^{2+}-Fluo-3 fluorescence in small hair initials of *Acetabularia acetabulum* (bar=75μm). (d)-(e) Gametophore growth in *Polyphysa peniculus* (bar=75μm). (d) Bright field photograph. (e) Intense Ca^{2+}-CTC fluorescence of the gametophores.

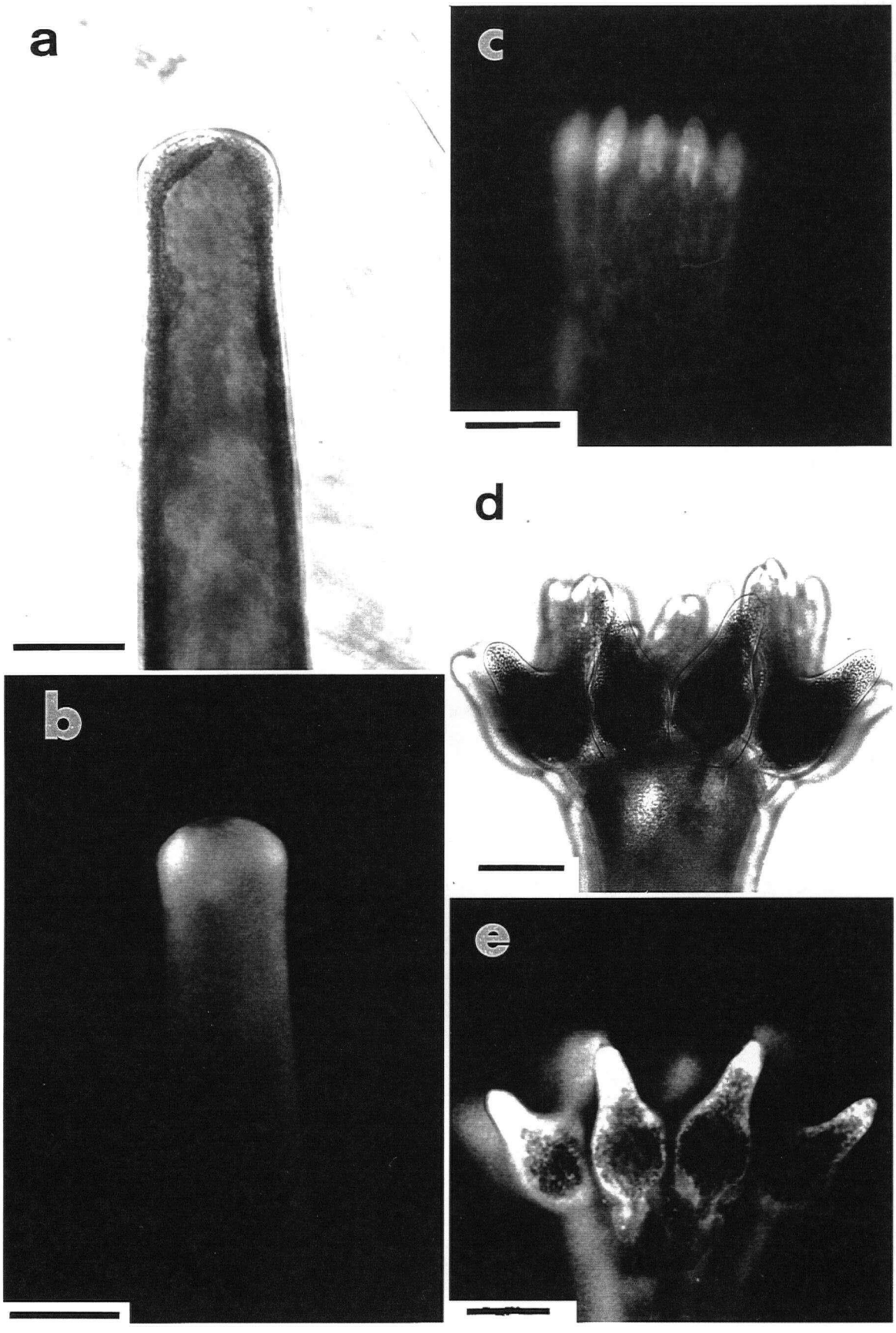


Figure 23

Figure 24: Fluo-3 staining. (a)-(b) Same cell as Figure 22b but this time stained with Fluo-3. The position of the initials is not seen as for CTC (bar=75µm). (c)-(d) Same cell as Figure 22c but this time stained with Fluo-3. The young cap contrast strongly with the remaining of the stalk. The injection point can be seen in the lower part of the image (bar=75µm).

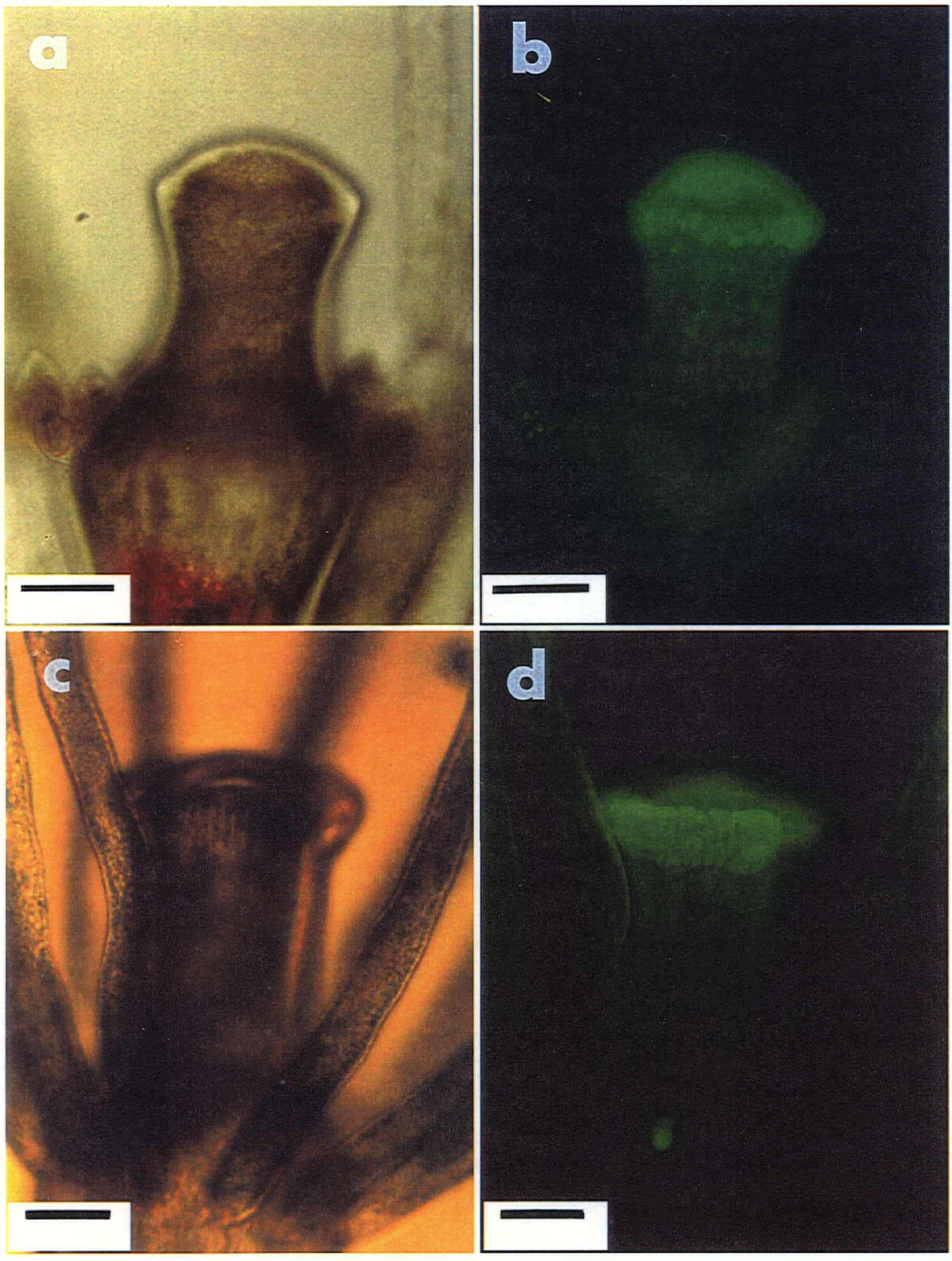


Figure 24

Figure 25: Fluorescein controls. (a)-(d) *Acetabularia acetabulum*. The fluorescein controls don't show the sharp contrast observed for Fluo-3.

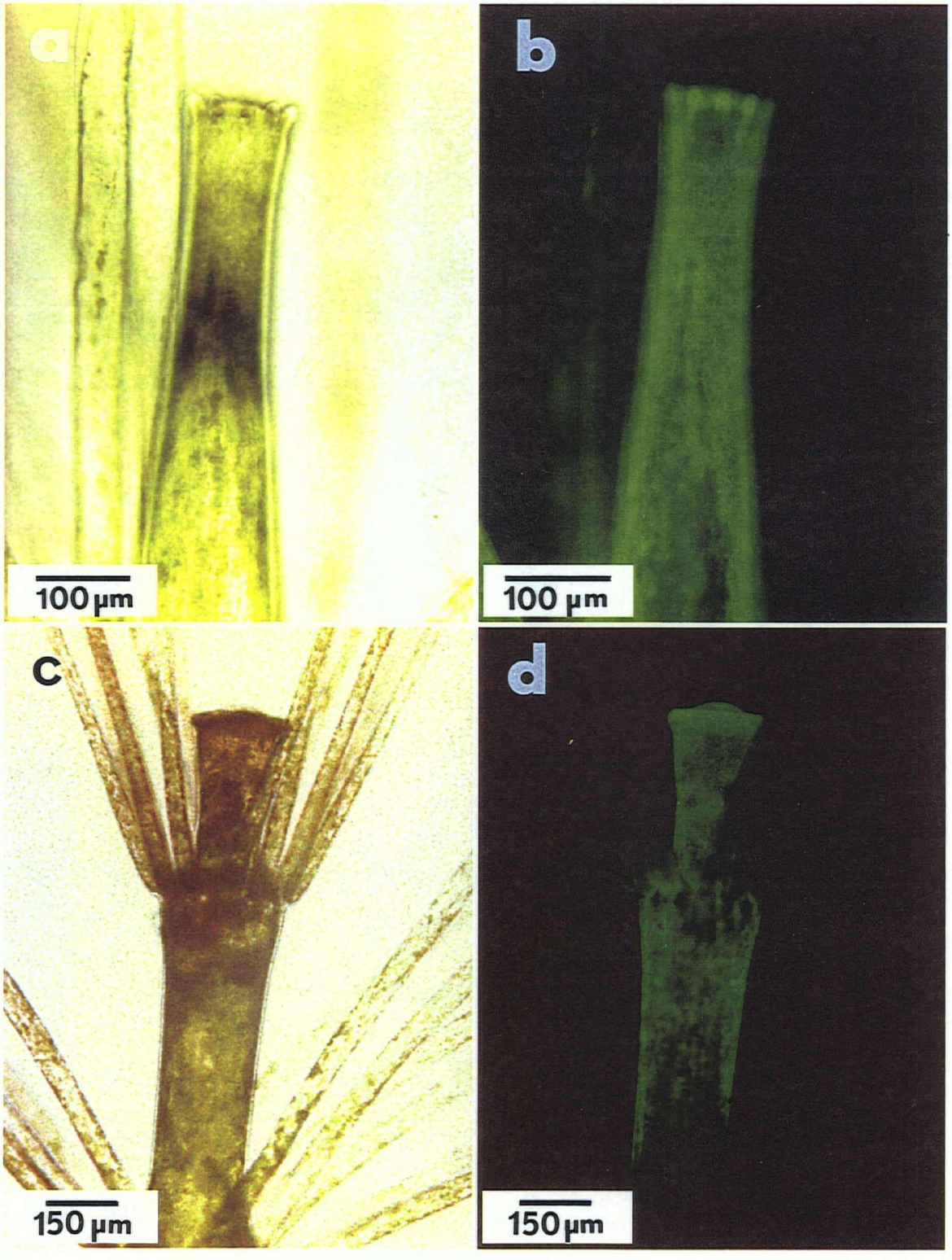


Figure 25

4.4 Discussion

The similar Ca^{2+} dynamics between the vegetative and reproductive whorls is compatible with the similarities found in their morphogenesis (see section 2). This is nevertheless different from the earlier report of a complete disappearance of Ca^{2+} fluorescence as soon as the cap is initiated (Cotton and Vanden Driessche, 1987). This discrepancy remains unexplained but I think I have provided sufficient evidence to suggest that membrane-bound and free cytosolic Ca^{2+} do have active roles in cap morphogenesis as for hair whorl morphogenesis. The results compare also relatively well with similar work in *Micrasterias* (Meindl, 1982). To my knowledge this is the only other unicellular organism showing branching tip growth for which membrane-bound Ca^{2+} has been labelled with CTC. Again the dichotomous branching of the growing tip and the distribution of Ca^{2+} along the cell membrane evolve in lock step with no evidence that Ca^{2+} ever forms a prepattern (based on the photograph provided by Meindl, 1982). The absence of prepattern undermines Goodwin's model but is compatible with the remaining models. The question raised in the introduction is left partly unanswered. It appears that the limited spatial and temporal resolution of the current techniques prevent us from making a stronger statement about the specific involvement of Ca^{2+} as a precursor or a second messenger (transducer) though its involvement as a morphogen is unlikely.

Explanations for these limitations are easy to find. The free cytosolic Ca^{2+}, believed to have such a tremenduous importance for the regulation of cellular processes, is present at very low concentration among large pools of bound, sequestered or extracellular Ca^{2+}. The specificity of the dye used and its uniform distribution are therefore critical to resolve accurately the distribution of free cytosolic Ca^{2+} (Tsien, 1994). Additionally, the distribution of free Ca^{2+} is labile, so that slight perturbations can lead to spurious results. Consequently, all experiments on free cytosolic Ca^{2+} need to rely on proper controls (see Read *et al*, 1992 for a discussion of this topic). The fluorescein controls conducted in this experiment test for the possible artifacts coming from uneven dye

distribution. A quick comparison between the controls and the Fluo-3 treatments demonstrates that the sharp contrast in fluorescence between the morphogenetic region and the rest of the cell arises from a genuine raise of Ca^{2+} in the morphogenetic region. To that extent, the results seem probative. Additional controls, perturbation with Ca^{2+} ionophores for example, would be useful to rule out other possible artifacts.

A different approach to the question might also be desirable. The cytoskeleton, membrane proteins and cytosolic proteins are possibly involved, along with Ca^{2+}, in the early stages of pattern formation. These constituants are more stable than Ca^{2+} and thus could provide a clearer picture, or at least a different picture, of the events taking place during morphogenesis. Menzel and Elsner-Menzel (1989, 1990) already described the complex involvement of the cytoskeleton during the differentiation of the cysts inside the gametophores. Similar studies have been done during the vegetative phase of the life cycle (Menzel, 1986) but to my knowledge they were never specifically aimed at the earlier stages of whorl formation. On the other hand, Werz (1959, 1965) has demonstrated the existence of "special proteins" with a distribution identical to the Ca^{2+} distribution described earlier (i.e., subapical annulus breaking up into a whorl pattern). Interestingly, these proteins seem to form an actual whorl prepattern since they precede the appearance of the initials and even the lysis of the wall. If these observations are correct, the unknown proteins could very well be one of the morphogen in a reaction-diffusion system.

Chapter 5

Higher plant morphogenesis: a lesson from the algae

This section has been inspired by Marius Chadefaud's paper, " La leçon des algues: comment elles ont évolué; comment leur évolution peut éclairer celle des Plantes supérieures" (Chadefaud, 1952). In his work, Chadefaud illustrates how the evolution of higher plants follows naturally from a major evolutionary trend observed independently in the Chlorophyceae, Rhodophyceae, Phaeophyceae, and Cyanophyceae algae. He argued not only for a natural continuity between algae and higher plants, he emphasizes also that the algae account for all the major structural innovations in the plant kingdom (e.g., the differentiation of rudimentary roots, leaves, flowers, and vasculature). This idea had already been recognized for the Dasycladales and the resemblance between the construction of *Acetabularia* (rhizoid-stalk-hair-cap) and the construction of higher plants (root-stem-leaf-flower) had been underlined by Nägeli (1847), Church (1895), Puiseux-Dao (1962, 1965) among others. Solms-Laubach (1894) and Church (1895) were able to draw an even closer parallel between hair and leaf based on two important observations. First, both organs show determinate growth, i.e., whenever a lateral is produced it will grow to a characteristic shape/length and then stop. Secondly, the hairs and the leaves are provided with a certain individuality which ultimately finds anatomical expression in the formation of perforated septa or the differentiation of an abscission zone. As a result, leaves and hairs are endowed with a deciduous habit.

These analogies in construction are readily recognizable and thus form the bulk of the literature on the subject (see Kaplan and Hagemann, 1991 for some general implications). Some authors went a step further and described to what extent the analogous structures shared common functions. For example, the anchoring role of the rhizoid and its capacity to accumulate reserve substances to withstand harsh conditions are similar in that respect to the functions of the root system in higher plants (Puiseux-Dao, 1962). But the analogy can hardly be extended further since the rhizoid

doesn't serve any absorptive function, an important feature of higher plant root systems. This latter function is fulfilled by the hair whorls in addition to their photosynthetic activity as shown by Gibor (see Gibor, 1989 for a review). He noted in cells kept in a neutral red solution that the dye uptake reveals a flow from the hair extremities to the central cavity (Gibor, 1973). Additionally, cells grown in a solution low in nutrients (e.g., low nitrogen) show a hypertrophy and a delayed abscission of their whorls (Adamich *et al*, 1975; Raven, 1986). This functional adaptation is known to occur in root systems growing in deficient soil or growth medium.

In most accounts I am aware of, the analysis stops at this point; i.e., the structure of fully developed organisms is compared, sometime the different functions are located, but rarely is the actual process of morphogenesis looked at comparatively. Even if this sort of comparative outlook would lead us to compare morphogenesis between unicellular organisms and multicellular organisms, the endeavour can be based on the fact that in both cases, morphogenesis is driven by an instance of *apical growth*, i.e., *tip growth* for the Dasycladales and *meristematic growth* for higher plants. So the question really is, what are the similarities in terms of pattern formation between these two instances of apical growth? A closer look at this question reveals several striking similarities.

1) Pattern formation occurs at similar scales (i.e., between 50 and 500 μm, 100 μm on average) despite the fact that the organisms involved might differ in final size by several orders of magnitude. For example whorl formation in *Acetabularia* occurs on tips ranging in size from 50 μm (first hair whorl) to 250 μm (cap).

2) Pattern formation along the main axis (stalk, stem) and the laterals (hair, leaf) is similar. The laterals are first formed in continuity with the main axis and they acquire a partial individuality ulteriorly when a boundary is established (septum and abscission zone). Despite the septum, whorl formation along the main axis and hair branching are very similar in the Dasycladales (see section

2.1). Yet, one important difference remains; the hair has a determinate growth while the main axis has an indeterminate growth. Similar observations were reported for higher plants: "On the basis of biometric analyses (Jeune, 1984a:118) concluded that the mechanisms of growth and branching are fundamentally the same in shoots and leaves, specifically with regard to the relation of growth in length and the rhythm of formation of the lateral elements." (Rutishauser and Sattler, 1985, p 437).

3) Pattern formation is influenced by the size of the apex, usually in a way suggestive of a characteristic wavelength for the pattern. For example, in *Acetabularia* (Harrison *et al.*, 1981) and *Equisetum* (Horsetail, Bierhorst, 1959) the number of laterals in a whorl is proportional to the whorl diameter when they are initiated (i.e., the spacing between the elements of a whorl tends to be constant). *Equisetum* and *Acetabularia* have also a similar variability in the number of laterals ranging from 3-6 in small whorls to 20-35 in large whorls (Harrison *et al*, 1981; Rutishauser and Sattler, 1987). For spiral phyllotaxis the rise in the number of parastichies as the tip increases in size would suggest again a fixed wavelength (Williams, 1975; Meicenheimer, 1979).

4) Development in algae and higher plants is heteroblastic, that is, the laterals vary in shape and function depending on their position along the main axis. This fact is well established for higher plants where the first two leaves formed (i.e., juvenile leaves) are usually different from the following ones (i.e., adult leaves) (Brink, 1962). Even among the so called adult leaves, a careful investigation often reveals a gradation in shape. Yet, the juvenile and the adult leaves are still recognizably leaf-like. The heteromorphose of the laterals is exacerbated in the floral parts (sepals, petals, stamens, carpel) where the identity with the leaves is much more subtle but now generally accepted. [The homology between the leaf and the flower parts was first recognized by the German poet and naturalist Goethe (see Arber, 1946 for an English translation of his work). Recent accounts can be found in Coen and Carpenter (1993) and Meyerowitz (1994)]. Interestingly a similar "maturation" of the apex morphogenesis can be found in multicellular algae (Lambert *et al*, 1995) and unicellular algae like the Dasycladales (Church, 1895; Nishimura and Mandoli, 1992).

In *Acetabularia*, the interwhorl length, the degree of branching in the whorl, and hair persistence show sharp discontinuities along the stalk, suggesting transitions between different stages of development (Nishimura and Mandoli, 1992). The cap of *Acetabularia* is an extreme case of such transitions.

These morphogenetic similarities are probably too numerous to be discredited as mere coincidences. At least two lines of explanation are possible. Church explained the structural analogies reported in terms of direct descent of the land plants from the green algae. He made his view clear in the very first lines of Thalassiophyta : " The beginnings of Botany are in the sea; and as it becomes more obvious that the vegetation of the land has at sometime originated from transmigrant marine phytobenthon, and that the somatic organization of branched cellular axes, stem and root, with apical growth and mechanism of leaf-arrangement, as also the entire phenomena of space-form, are the inherited equipment of a preceding phase of existence in the wholly submerged environment of the sea..." (Church, 1919 p 3). Moreover, Church would add: " The cells and somatic organization of all land-plants, as also all their reproductive cycles and mechanism, are but the continuation of the mechanisms evolved in the sea, to suit the conditions of life in the sea, as the best response possible under such conditions; and though the mechanism may be emended, modified, or superseded in innumerable details, the primary plan of the architecture, and the entire range of general principles of organization, remain essentially marine." (Church, 1919 pages 91-92). Following Church, the mechanism of morphogenesis would have evolved under selective pressures of the sea environment and then be preserved, in essence, in the following evolution of higher plants, hence the observed similarities in morphogenesis. This was certainly his view about the mechanism of phyllotaxis: "... one is justified in concluding that Fibonacci phyllotaxis was initiated in the sea...although in transmigrant land-vegetation the system of construction may prove valuable under the new conditions, and so be retained as one of the most deeply ingrained construction-factor of the leafy shoot. It being so far clear from the organization of the Fucaceae that Fibonacci relations are older phylogenetically than the

differentiation of the 'leaf' itself as a strict morphological entity (as defined in terms of subaerial vegetation)"(Church, 1968). Similar views have been expressed elsewhere (Chadefaud, 1952; Emberger, 1968).

In recent years some authors have taken a different stand on this question. For example, Goodwin (1990) disagrees with the functional explanations as used by Church, principally because these explanations are *ad hoc*. He proposed a structuralist view of this question, i.e., if a specific development has appeared in the green algae like the Dasycladales, it is because it represents a stable dynamic (an *attractor*). The persistence of this specific development in higher plants would be attributed not so much to descent but to the fact that they obey the same "biological law(s)".

Whether Church's or Goodwin's explanation is prefered, the study of algae is shown to be relevant to the understanding of morphogenesis of higher plants. Is it possible that these analogies in morphogenesis indicate that the pattern forming mechanism has been preserved in the evolution from green algae to the land flora? Is it possible that both groups respond to a common attractor? It would be presumptuous to answer these questions now since conclusive evidence for a specific morphogenetic mechanism has yet to be found in either of these groups. Interestingly, the three models addressed in this work are paralleled by similar models for the morphogenesis of higher plants. Models for higher plant phyllotaxis have been based on mechanical buckling of the tunica (Green, 1992), reaction-diffusion (Turing himself, see Saunders, 1992; Meinhardt, 1982; Berding *et al*, 1983) and mechano-chemical interaction involving this time diffusion of a chemical inhibitor and contact pressure between the primordia (Roberts, 1978). Therefore the empirical observations and the theoretical background show enough similarities to warrant an attempt to learn from work done on both group of organisms. Hence, what lessons can be learned from the algae? Two facets of the pattern formation viewpoint have proven to be useful in their application to the Dasycladales.

Firstly, pattern formation needs to be studied as it unravels. Some of the most interesting features of whorl formation like wall lysis (Werz, 1965), the complex dynamics of Ca^{2+} distribution (Harrison *et al*, 1988) and the important homologies between the vegetative and reproductive whorls (Valet, 1968) have been found in this way. The morphogenetic work done in the Dasycladales and higher plants is not different in that respect but the importance of studying the patterns as they arise needs to be restated periodically. For instance Sachs made recently such a statement in relation to the regulation of stomatal patterning on leaves: " The purpose here will be to point out that the critical facts could only come from the way the patterns are formed during development, not from mature structures" (Sachs, 1994).

Secondly, the dynamical approach to morphogenesis (see section 1.2) is underdeveloped for higher plants. To my knowledge, the quantitative test of the regulation of λ_s hinted by Martynov and successfully performed by Harrison (see section 1.3) has yet to be emulated by research in higher plants, this despite the many opportunities available. One such opportunity is whorl formation in *Equisetum.* A quote from an early paper still characterizes very well the spirit in which many studies are still done: " In *Equisetum* the number of leaves initiated within a given whorl is a function of the size of the shoot apex at the time the leaves are initiated. This is so obvious from superficial examination of transverse and longitudinal sections of shoot tips that the presentation of refined quantitative data could add nothing" (Bierhorst, 1959). Over two sentences, an important structural feature, that is, the constant spacing suggested by the observations, has been discarded because judged too trivial to deserve additional work. In my view, the "refined quantitative data" on the regulation of these structural features is actually what is too often missing in the work done on morphogenesis.

Chapter 6

Conclusion and future research

Even if the conclusions about whorl formation in the Dasycladales are limited, I think this work shows how different prespectives can interact positively to produce a clearer understanding of morphogenesis.

The attempts made in this work to find a chemical or a mechanical "prepattern" show one limitation of the structural approach, that is, the cause and the effect are difficult to resolve from each other. For example the staging of section 2.1 suggest that wall lysis precedes wall bulging but the amplitude predicted by the buckling mechanism are so small that their presence cannot be surely ascertain and the evidence that buckling is not leading morphogenesis is weaken. Similarly, the results sofar suggest that neither bound or free Ca^{2+} form a prepattern before structural differentiation but again the two events are so close in time that a decisive answer cannot be given. It seems that chemistry and structural changes are so tightly tied together in algae and higher plants that all attempts to find which one come first can only be suggestive of their order of appearance. Decisive experiments need to be found elsewhere. The dynamical approach could certainly provide new opportunities for such experiments. The value of testing the regulation of spacing by different factors has been underlined already. Along similar lines, manipulation of the boundary conditions of the domain where pattern formation is occuring could yield interesting results. These manipulations have been achieved recently using diffusion barriers (Siegel and Verbeke, 1989) and mechanical constraints (Green, 1993). The two types of dynamical approach have a common requirement, that is, they need to be based on a paradigm or a morphogenetic model to suggest variables to test and a proper interpretation of the results.

Comparative approaches provide also useful information on the intraindividual, intraspecific and interspecific variation of morphogenesis. These evidences can be used to discriminate between

what is essential to morphogenesis and what is simply peripheral. It appears also that comparison between seemingly distant groups like the algae and higher plants could unravel significant similirities in their morphogenesis; therefore theories and experiments need not to be confined to one's specific object of research. Numerous oppotunities for future research are available for all approaches of morphogenesis.

Structural approach

Important work remains to be done with the structural approach even if the search for a prepattern in might not itself yield conclusive evidence on the nature of the morphogenetic mechanism (chemical, mechanical, mechano-chemical). Clearly the work undertook by Werz (1965) on the wall lysis could be supplemented by additional research on membrane proteins, the cytoskeleton and wall chemistry. One of these cellular components is likely to be responsible for the beautifully ordered punctate lysis described in section 2.1. The wall lysis is in itself the structural expression of similarly patterned chemical reactions. Characterizing these would move the structural analysis on step closer to pattern formation.

Additionally, the morphogenetic differences found between the hair and the gametophore and between *Halicoryne* and the other Dasycladales would benefit from close structural observations. A clearer understanding of the differences involved would tell us if variation of one mechanism is sufficient to explain the range in morphogenesis observed.

Dynamical approach

This approach is so underdeveloped in biology that almost everything remains to be done. Additional testing of the regulation of spacing in the whorl is still very attractive. The punctate lysis would enable measurements before the appearance of the initials and a spacing value could now be obtained for each initial in the whorl instead of one average value for the whole whorl. In these

conditions, the most critical experiment for wall buckling, i.e., the effect of wall thickness on the spacing, could be readily tested with a very good accuracy.

Theoretical approach

The morphogenetic sequence presented in section 2.1 is to a large extent outside the reach of current modelling. This begs the question of what would be an appropriate model of Dasycladalian morphogenesis? Following Church: "the archetype of the Dasycladaceae may...be conceived to have consisted of a main axis bearing whorls of several times polytomizing foliar appendages" (Church, 1895, page 593). The discussion of section 3.3 reveals that the extant species of Dasycladaceae and Acetabulariaceae are variations of this archetype. Whorl formation during the vegetative phase of the life cycle of *Acetabularia* is therefore a very appropriate for the first modelling attempts. Computations by Harrison *et al.* (1981) and Goodwin and Brière (1994) have already shown a very good approximation of whorl formation but a complete 3-D model is still awaited.

Numerous biological observations remain to be included in the morphogenetic mechanism. For example two major observations on photomorphogenesis have yet to be addressed by the different mechanisms. First, Gibor's finding (see his 1989 review) that illumination of the tip is sufficient for growth and morphogenesis while illumination of the whole cell except the tip is not enough to ensure growth. Second, cells grown in red light grow apically without forming whorls. In these conditions, a flash of blue light is sufficient to resume morphogenesis (Schmid *et al*, 1987). Also the classical experiments made by Hämmerling (1963), which uncover the involvement of long lived mRNA in species specific morphogenesis, have still to be included in the current morphogenetic mechanisms.

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