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Morphogenetic oscillations during symmetry breaking of regenerating *Hydra vulgaris* cells

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Abstract. – During the process of regeneration, the fresh-water polyp *Hydra vulgaris* first builds a hollow sphere consisting of a cell bilayer. This cell ball undergoes subsequent shape transformations, at a later stage it creates tentacles and a foot to form an animal. We describe and analyze the transformation of the hollow sphere to the first non-spherical shape by means of contour analysis. We observe that the cell ball shows characteristic oscillations in size and shape which accompany symmetry breaking. Quantitative analysis of these oscillations provides information on the cell bilayer mechanics and hydrodynamic flows involved. In order to explain the origin of the observed oscillations, we propose three different physical scenarios of oscillation generation.

Introduction. – During development, clusters of initially undifferentiated cells undergo transformations that lead to patterns [1] and complex morphologies of differentiated cells. Initial steps of development are similar in different species, pointing to similar mechanisms as an underlying building principle. The strategies which govern this process and the regulation mechanisms which are involved in cell differentiation are subject of intense studies [1–7]. Here we describe certain macroscopic observations during development and we highlight possible mechanisms generating the observed behaviour.

Turing suggested that a set of morphogens which determine the fate of undifferentiated cells is transported via diffusion and undergoes biochemical reactions [7]. These ideas were further developed by Gierer and Meinhard [8, 9] and were very fruitful for the understanding of the observed stability and the regeneration capabilities of hydra, although the messenger molecules which were postulated have not been identified so far.

Hydra vulgaris is a fresh-water polyp, one of the oldest still existing multicellular organisms. An adult *Hydra vulgaris* is up to 15 mm long and can be found in tap water in remote unpolluted regions (fig. 1). It mainly consists of a hollow tube, the "gastric column", made of an inner and an outer cell layer. The head is equipped with a mouth opening surrounded

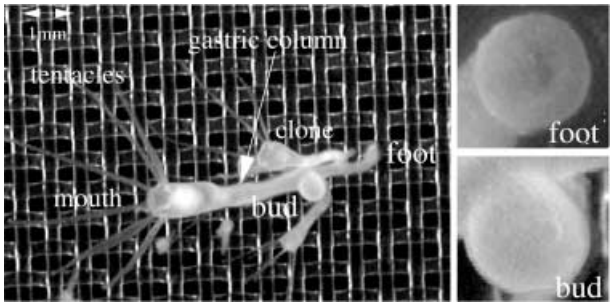

Fig. 1 – *Hydra vulgaris* is a fresh-water polyp consisting of three main parts: a mouth with tentacles, a gastric column and a foot. The cell wall is composed of two cell layers separated by a membrane. Reproduction by budding is the most common, however, creation of eggs could also be observed.

by typically 3–6 tentacles. A foot [10] enables hydra to attach to a substrate. The ability of *Hydra vulgaris* to regenerate is spectacular, *e.g.*, a few thousand gastric hydra cells, even as a disorganized cluster, may regenerate into an adult animal without relation to their former function and position [11]. In contrast, the mouth, the tentacle and the foot cells are irreversibly specialized [12].

At the beginning of the regeneration process, cells arrange to form a hollow ball. This ball slowly elongates, then starts to grow tentacles at one end and finally transforms to a fully developed animal. We study the dynamics of the initial steps of morphology changes of hydra when the hollow sphere loses its spherical symmetry. It elongates and subsequently the mirror symmetry with respect to the midplane perpendicular to the remaining rotational axis is broken. This symmetry breaking is accompanied by relaxation oscillations of the ball radius [13, 14]. In the following, we provide a systematic and quantitative analysis of the shape of the cell ball as a function of time. We characterize three different types of oscillations which anticipate and accompany the symmetry breaking of the initially undifferentiated sphere. Furthermore, we propose three different physical scenarios which can lead to these types of oscillations.

Materials and methods. – The preparation of hydra tissue follows mainly the description in [15]. Hydras are raised in Volvic mineral water [16]. Non-budding animals with a length of approximately 7 mm are used for experiments after they are starved for 24 h.

Using a surgical knife, a small ring of tissue is cut out of the middle part of the gastric column under a stereoscope (Zeiss Stemi 2000). This ring is then opened with two axial cuts and the so-formed small squared tissue sheets of about 300 μm are immersed in culture solution. After 3–4 hours, regular hollow spheres form. The hydras are embedded into a very soft agarose gel (0.02 g/10 mℓ), providing stability without generating relevant counter-forces. These spherical cell balls are observed under a self-assembled microscope equipped with 5× objective (ZEISS Plan-Neofluar 5X/0.15 NA), a CCD camera (Cohu 4900) and a timelapse VCR (Sanyo). The pictures on the videotape are digitized with 320 × 240 resolution on a Silicon Graphics O_2 workstation. Analog and digital noise is suppressed by smoothing (3 × 3 pixel averaging).

We observe the deformations only in the plane of observation of the microscope, hence, in the case of coincidence of the organism-axis with the axis of observation, the deformation would be ignored. However, gravity working in our favour, this case was found to be uncommon. We determine the contour (algorithm will be published elsewhere) of which we discuss the dynamics of the 0th (averaged radius) R_0 and 2nd (elongation) Fourier modes R_2 in a semi-discrete

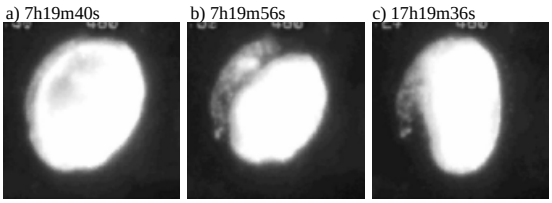

Fig. 2 – The hollow cell ball (about 10^4 cells, diametre: 200 μm) before (a) and after (b) a contraction, (c) the shape becomes oblong at a later stage of regeneration. Time scale refers to figs. 3 and 4.

polar coordinate system with 32 equidistant angles ϕ_m: $R(\phi_m) = R_0 + \mathrm{Re}[\sum_{n=1,2,\ldots} \tilde{R}_n e^{in\phi_m}]$, The position of the coordinate system is such that the 1st mode disappears.

Results. – Cell balls prepared as described above fold within 3-4 hours to form a hollow sphere. It grows and collapses repetitively as shown in figs. 2, 3 and 4. The observed periodic expansions and contractions can be distinguished (figs. 3 and 4) from active motion of adult

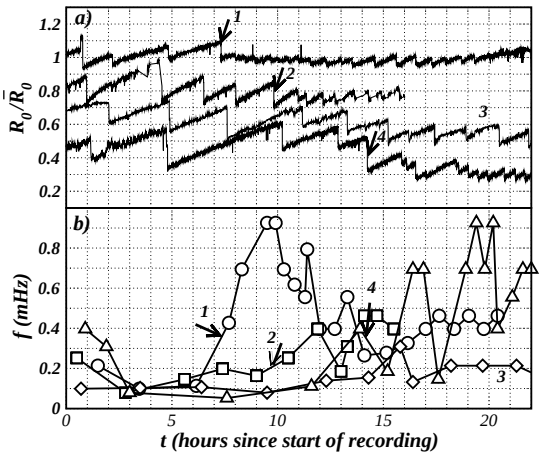

Fig. 3

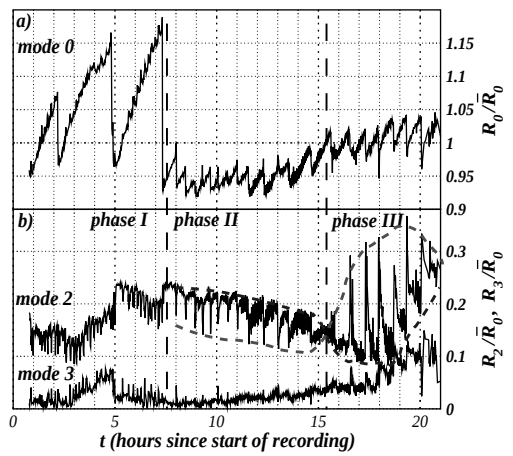

Fig. 4

Fig. 3 – a) shows the sphere radius R_0 scaled with its mean value over the observed interval as a function of time for 4 different hydra balls (curves 1–4). The curves 2, 3, 4 are staggered for clarity. The transition from large- to small-amplitude sawtooth oscillations is indicated for curve 1, 2 and 4. Curve 3 will not lead to successful regeneration. b) The corresponding frequencies of the sawtooth curves have been determined by calculating the positions of the sawteeth using a multi-scale analysis with a sawtooth test-function. The frequency is the inverse of the position differences of respective neighbouring sawteeth.

Fig. 4 – The scaled radius R_0, elongation R_2 and triangular deformation R_3 of the contour of sample 1 in fig. 3a) are displayed. The scaling factor is the average $\langle R_0 \rangle_t = \bar{R}_0$ over the whole time interval. Three different phases can be distinguished: phase I (t = 0–7.4 h), phase II (t = 7.4–15.5 h) and finally phase III; the reversal of the peaks of the second mode between phase II and III defines the beginning of phase III: the intersection of the envelopes (dashed lines) of the second mode. The radius oscillations of the sphere remain identical to phase II. Mode 3 grows during phase III as an indication of more complex structures emerging. At the end of the recording, the curves become noisy and ill-defined due to the development of an emerging 3D morphology which is not well represented by a 2D contour.

hydras [17, 18], which, in contrast, are very sensitive to mechanical disturbances and even to ambient illumination [17].

The 14 hydras we analyzed exhibit sawtooth oscillations. Throughout the observation, the inflation of the hollow cell ball is characterized by a scaled growth rate $\dot{R}_0/\bar{R}_0 \approx 2 \times 10^{-5}\,\mathrm{s}^{-1}$. During the interval of observation three phases of characteristic oscillations can be distinguished (figs. 3 and 4):

Phase I shows low-frequency relaxation oscillations with a period of 1–3 hours. They are characterized by a slow constant inflation of the sphere followed by rapid contractions as soon as some threshold is reached. During these oscillations, the radius changes by up to 20%. During rapid shrinkage, it can be seen that the cell ball bursts occasionally and releases fluid from the inside together with detached cells [14] (fig. 2). This indicates that pressure builds up inside the ball when it inflates, which is also supported by the observation that the second mode R_2, measuring the elongation of the organism, often increases during deflation. This is consistent with an isotropically elastic ball which when inflated becomes more spherical.

Phase II sets in abruptly after 7 to 13 hours of observation. The system continues to oscillate, however with four times higher frequency and smaller amplitude (figs. 3 and 4). The elongation (second mode) fluctuates synchronously to the contractions of the radius. During this phase, the degree of elongation decreases upon deflation. This behaviour is not clearly apparent at the beginning of phase II but becomes gradually more pronounced. The observation that the shape becomes more elongated as it inflates indicates that the symmetry is broken since the elastic properties of the ball are non-homogeneous on the sphere. We therefore conclude that the symmetry of the cell ball is being broken at the beginning of or during phase II.

Phase III sets in several hours after the onset of phase II. It is characterized by a new behaviour of the second mode R_2. While the radius oscillations R_0 stay the same as during phase II, the elongation R_2 increases strongly while the ball contracts, and then relaxes slowly to a more spherical shape upon inflation. These observations suggest active deformation of hydra, faster than the time resolution of our experiment (*i.e.* 54 s) possibly due to contractile elements in the cells. Maximal elongation is maintained only shortly (shorter than a minute), relaxation towards a spherical shape is slow, on the order of a few tens of minutes. The sawtooth oscillations of the radius remain unchanged with respect to phase II, they take place simultaneously with respect to elongation.

Discussion. – The observed oscillations (figs. 3 and 4) of the radius exhibit non-linear relaxation oscillations [19] characterized by a slow and steady inflation of the ball until a rapid contraction occurs when a threshold radius is reached. These contractions are about 500 times faster than inflation. The contractions of the radius during phase II and III are of four times lower amplitude, indicating that the threshold mechanism has changed. The constant inflation rate throughout the whole observation period and for all hydras we studied suggests that inflation is always due to a constant influx of fluid into the sphere during all three phases. Our precision is not sufficient to detect the deviation from a linear slope, which would be caused with a constant influx of fluid and the cubic ratio between volume and radius. We assume that this flux of hydra medium is driven osmotically by cellular ion pumps which actively transport ions into the ball.

The hydrodynamic flows during inflation and the mechanical stresses induced can be estimated as follows: The initial diameter of the cell ball is approximately. $\bar{R}_0 \simeq 200\,\mu\mathrm{m}$, the internal volume is $4\,\mathrm{n}\ell$. With an inflation rate of $\dot{R}_0/\bar{R}_0 = 2 \times 10^{-5}\,\mathrm{s}^{-1}$, the inflation is accompanied by a volume change of $\dot{V} = 3V\dot{R}/\bar{R}_0 \approx 10^{-7}\,\mu\ell/\mathrm{s}$ and by an inflow of fluid through the cell wall of the order of $J_0 = \dot{V}/A_0 \approx 10^{-8}\,\mathrm{m/s}$. We assume that this flux of hydra medium is driven osmotically by pumps which actively transport ions into the ball.

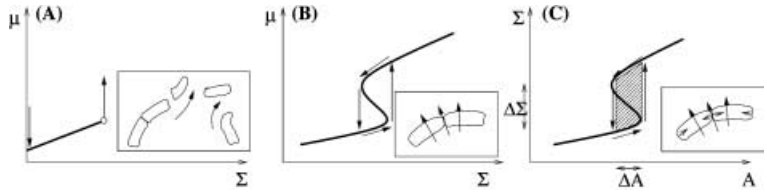

Fig. 5 – General mechanisms for relaxation oscillations. Inflation is due to a constant influx of fluid driven by the active pumping of ions. Three different scenarios could describe the instability leading to relaxation oscillations: (A) rupture of the cell layer, (B) cooperative channel opening, and (C) active contraction.

The osmotic pressure of the ions balances the pressure difference $\Delta P \simeq 2\Sigma/R_0$ due to elastic deformation of the cell ball and the resulting mechanical tension $\Sigma \simeq \chi(A - A_0)/A_0$. Here, $A - A_0$ denotes the elastic area increase and χ the area-elastic modulus of the cell ball. The elastic modulus $\chi \approx 10^{-3}\,\mathrm{N/m}$ is estimated from observing elastic deformation of the ball after applying a force using a glass micro-plate of known elasticity [20].

Using the observed radius change of 20%, we estimate $(A - A_0)/A_0 \simeq 0.44$ and $\Sigma \simeq 10^{-2}\,\mathrm{N/m}$. With $\Delta P \simeq 2\Sigma/R_0$, the osmotic pressure is of the order of $\Delta P \approx 10\,\mathrm{Pa}$. The corresponding concentration $C = \Delta P/(N_\mathrm{A} k_\mathrm{B} T)$ of excess ions in the cell ball is estimated to be $C \simeq 4\,\mu\mathrm{mol}/\ell$, *i.e.* approximately 10^{10} ions at room temperature. In order to achieve the above estimate for the volume flow, pumping rates of 10^6 ions/s are required. Given that active ionic pumps could carry up to several hundred ions/s [21], 10^4 pumps or a few pumps/cell would be sufficient to account for the observed phenomenon.

The volume inflow due to active pumping is counteracted by passive outflow. We therefore write the total flow as $\dot{V} = J_0 A_0 - \Delta P \mu A_0$, where μ denotes the passive permeability of the cell layer to ions which in general is a function of tension Σ. As long as the second term is small compared to the first one, the ball inflates. In order to generate relaxation oscillations, there must exist a mechanism that provides for rapid relaxation and, thus, an outflow of ions as soon as a threshold of the radius is reached. Such a threshold could most naturally be realized by a sudden increase of the permeability $\mu(\Sigma)$, as soon as a critical tension Σ_c is reached.

We distinguish three different mechanisms which could lead to relaxation oscillations (fig. 5(A-C)): (A) Rupture: when the osmotic pressure induces a mechanical tension reaching a critical value Σ_c for which the cell bilayer ruptures, the opening of a small hole leads to a sudden increase in permeability μ and thus a rapid deflation driven by osmotic pressure. (B) Mechano-sensitive ion channels could have non-linear opening-*vs.*-tension characteristics and open collectively at a critical tension, releasing the inner pressure. This would lead to rapid deflation as in (A), however typically at a different tension threshold. (C) Active contractions: Active tension could be generated by cells in the ball due to cellular contraction mechanisms. This could also lead to an increase in pressure, expelling the inside fluid. Such a contraction could be triggered as soon as a critical amount of cellular deformation proportional to $A - A_0$ is reached.

During the contractions in phase I, we observe an expulsion of material and cells from the ball, in agreement with [14]. This observation suggests that the cell wall ruptures during deflation, indicative of scenario (A). The idea of leakage through a hole in a rupturing cell ball has many similarities with the dynamics of vesicles with opening and closing of vesicle pores [22]. The volume flow of the leaking fluid is related to the flow velocity v_L via $\dot{V} \simeq v_\mathrm{L} d^2$. We can obtain an estimate for the hole diameter assuming that a single hole of size d opens,

assuming that v_{L} scales as $v_{\mathrm{L}} \simeq d\Delta P/\eta$. During rapid relaxation, $\dot{V} \approx 10^{-4}\,\mu\ell/\mathrm{s}$. With the above estimate for the pressure ΔP, we find $d \simeq (\dot{V}\eta/\Delta P)^{1/3}$ to be of the order of $d \approx 10\,\mu\mathrm{m}$, which is of the order of the size of hydra cells. In this simple model, the flux through the membrane is proportional to the pressure difference across it. Therefore, the relaxation is expected to be exponential in this case.

After the sudden transition to phase II, relaxation oscillations have the same inflation rate as before, but the threshold at which deflation occurs is reduced. Since deflation in phase II occurs at lower tension, we suggest that a new mechanism for sudden leakage is now available. A possibility is the scenario (B) described above, where coordinated opening of mechanosensitive ion channels or loosening cell junctions could lead to an instability. Looking at the symmetry breaking mode R_2 during phase II, we find that toward the end of this phase R_2 shows an increasingly pronounced growth during inflation. The fact that with increasing pressure the shape becomes more elongated indicates that the poles defined by the long axis of the organism are of reduced elasticity relative to the equator elasticity. This shows that the elastic properties of the cell ball break the spherical symmetry. Note that the relaxed shape remains close to spherical. Thus, symmetry breaking of the mechanical properties of the cells on the sphere occurs at the beginning of or during phase II.

At the onset of phase III, the behaviour of the elongation mode R_2 changes. The mode increases during the relaxation phase and the cell ball slowly becomes more spherical during inflation. The increase of R_2 during relaxation is an indication that at the onset of phase III the preferred shape of the ball is no longer a sphere but has broken symmetry. Under pressure increase during inflation, this relaxed shape is deformed in a more spherical shape. Observation of the video sequences of shape changes during this phase gives the impression that contractions are active. This is supported by statements of Sato-Maeda *et al.* [23] and corresponding to scenario (C). However, the possibility that the elongations are due to rapid pressure decrease in a non-spherical cell ball (scenario (B)) cannot be excluded.

Conclusion. – We report a quantitative study of the dynamics of shape changes of a spherical ball of hydra cells at the first stages of development by regeneration using a newly developed contour-retrieval algorithm giving access to the corresponding Fourier modes. We observe sawtooth oscillations (relaxations oscillations) of the radius. The fact that the growing slope of the sawteeth remains unchanged throughout our observation suggests that a common transport mechanism is at the origin of inflation of the cell ball.

For the interpretation of the contractions we propose three different mechanisms of nonlinear instabilities. It is plausible that all three mechanisms are implicated in the sequence of events that is precisely regulated. The oscillations of shape go through three well-identified phases of motion.

Our observations show that morphological symmetry breaking always occurs after completion of phase I. It appears as an inhomogeneity in the elastic properties of the spherical cell ball. This suggests that the described mechanical oscillations are an integral part of the developmental mechanism. This is corroborated by the fact that all hydras which do not show relaxation oscillations fail to regenerate. Hydras which do regenerate have in common the intense and well-regulated mechanical activity during regeneration which evolves in a well-defined way via the three phases characterized. The periodic shape changes and inflation of oscillations could provide a synchronization of cell states along the cell ball and a time reference for developmental changes. Further research needs to place these results into the molecular context of cell signaling and pattern formation: It will be interesting to see if the emergence of the hydra WNT signalling cascade, as observed in [24], coincides with the end of phase I, pointing further to a link of the observed characteristic mechanical motion and axis establishment.

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