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Cooperative differentiation through clustering in multicellular populations

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Abstract

The coordinated development of multicellular organisms is driven by intercellular communication. Differentiation into diverse cell types is usually associated with the existence of distinct attractors of gene regulatory networks, but how these attractors emerge from cell-cell coupling is still an open question. In order to understand and characterize the mechanisms through which coexisting attractors arise in multicellular systems, here we systematically investigate the dynamical behavior of a population of synthetic genetic oscillators coupled by chemical means. Using bifurcation analysis and numer-

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ical simulations, we identify various attractors and attempt to deduce from these findings a way to predict the organized collective behavior of growing populations. Our results show that dynamical clustering is a generic property of multicellular systems. We argue that such clustering might provide a basis for functional differentiation and variability in biological systems.

Key words:

Multicellular systems, clustering, collective behavior, inhibitory cell-to-cell communication, cellular differentiation

1 1. Introduction

2 The coordinated behavior in multicellular systems results from a coop-
3 erative response arising from an integrated exchange of information through
4 cell-cell communication. Various mechanisms for intercellular coupling have
5 been identified in nature, basically relying on the broadcasting of individ-
6 ual cellular states to neighboring cells via intercellular signals, which are
7 further integrated to generate a global system's response (Heinlein (2002);
8 Perbal (2003)). It is known, for instance, that bacteria display various types
9 of collective behavior driven by a type of chemical cell-cell communication
10 mechanism known as quorum sensing (Taga and Bassler (2003)). This ability
11 of living systems is an absolute requisite to ensure an appropriate and ro-
12 bust global cellular response of an organism in a noisy environment. Hence,
13 characterizing the dynamics of multicellular systems should lead to an im-
14 provement of our knowledge about cellular behavior and biological mecha-
15 nisms that occur on a population-wide scale, such as cellular differentiation,
16 adaptability of the system to different environment conditions, etc.

17 In order to understand the basic mechanisms of cell-to-cell cooperative be-
18 havior, several theoretical models have been successfully developed and inves-
19 tigated by studying both natural and synthetic genetic networks (McMillen
20 et al. (2002); Taga and Bassler (2003); Kuznetsov et al. (2004); García-Ojalvo
21 et al. (2004); Ullner et al. (2007); Balagadde et al. (2008); Tanouchi et al.
22 (2008)). A synchronization scheme has been proposed, for instance, in an
23 artificial network of synthetic genetic oscillators that produces and responds
24 to a specific, small signaling molecule (acylated homoserine lactone), known
25 as an autoinducer (*AI*) (García-Ojalvo et al. (2004)). This small molecule is

26 free to diffuse through the cell membrane, which provides a means for chem-
27 ical communication between neighboring cells. The resulting synchronized
28 behavior leads to a macroscopic genetic clock. By further manipulations of
29 this synthetic network, we were able to show (Ullner et al. (2007, 2008))
30 that this communication scheme can be re-engineered to produce a very di-
31 verse dynamics and exhibit a high adaptability typical to natural systems.
32 Other modification of the same network (García-Ojalvo et al. (2004)) was also
33 published recently (Zhou et al. (2008)), but it still waits further dynamical
34 investigations.

35 In this paper, we investigate systematically the global cooperative behav-
36 ior of a population of synthetic genetic oscillators called repressilators, cou-
37 pled via quorum sensing mechanism (Ullner et al. (2007)), and thus showing
38 the emergence of a rich variety of clustering behavior that might be inter-
39 preted as a mechanism of dynamical differentiation. Such an interpretation
40 was pioneered by Turing (Turing (1952)) in his investigations of inhom-
41 ogeneous steady states in reaction-diffusion systems, and has been further
42 extended by Kaneko (Kaneko and Yomo (1994)), who proposed clustering in
43 coupled map dynamics as a physical background of biological differentiation.
44 Moreover, it was recently shown (Nakajima and Kaneko (2008)) that bifur-
45 cations driven by cell-cell interaction may mediate differentiation processes.

46 Here we use numerical simulations and bifurcation analysis to study the
47 dynamics of a population of coupled genetic oscillators for increasing sizes
48 of the population, ranging from a two-cell to a multicellular system, thus
49 proposing a general explanation for the emergence of cooperative behavior
50 in large cellular systems. In our previous work (Ullner et al. (2007, 2008))

51 we investigated and defined the necessary coupling conditions leading to a
52 complex dynamical behavior in the system, and furthermore clasified its dy-
53 namical structure using a minimal system of $N = 2$ oscillators. In addition to
54 those earlier results, here we show that the extended system exhibits various
55 attractors with complex phase relations, and through their characterization
56 we attempt to (i) deduce the underlying mechanism that determines the most
57 likely visited dynamical regimes, and (ii) identify stable cluster distributions,
58 in order to predict the behavior of the system on a global scale. The bifurca-
59 tion analysis and numerical investigations presented here also aim to charac-
60 terize the robustness of the dynamical structure of the system with respect
61 to parameter variations, and relate these findings to biological processes. We
62 stress here the importance of investigating dynamical clustering in multice-
63 lular populations with cell-cell communication, since such coupling generates
64 qualitatively new cellular states different from the single-cell dynamics, thus
65 providing the basis for functional differentiation and variability.

66 Moreover, in (Ullner et al. (2008)) we identified a biologically relevant
67 parameter interval where chaotic behavior of the coupled genetic units was
68 observed. As previously suggested, this could implicate chaos as an addi-
69 tional source of uncertainty in gene expression, drawing attention on possi-
70 ble alternative sources of uncertainty in genetic networks, besides the already
71 well-established ones. It is therefore important to investigate the dynami-
72 cal behavior of cell populations in the chaotic regime, and identify possible
73 groupings of genetic oscillators and their relations within, as a base for en-
74 visioning an experimental protocol to detect chaotic behavior in synthetic
75 genetic networks.

76 **2. Model of a synthetic multicellular system**

77 The model considered here consists of a population of repressilators cou-
 78 pled via quorum-sensing mechanism as proposed in (Ullner et al. (2007)).
 79 The repressilator consists of three genes whose protein products repress the
 80 transcription of each other in a cyclic way (Elowitz and Leibler (2000)). In its
 81 original experimental implementation, the gene *lacI* expresses protein *LacI*,
 82 which inhibits transcription of the gene *tetR*. The product of the latter, *TetR*,
 83 inhibits transcription of the gene *cI*. Finally, the protein product *CI* of the
 84 gene *cI* inhibits expression of *lacI* and completes the cycle (see Fig. 1). An
 85 additional feedback loop involving the two proteins *LuxI* and *LuxR*, which
 86 might be placed on a separate plasmid, realizes the cell-to-cell communication
 87 (McMillen et al. (2002); You et al. (2004); García-Ojalvo et al. (2004)). *LuxI*
 88 is responsible for the biosynthesis of a small signaling molecule, known as au-
 89 toinducer (*AI*), which diffuses through the cell membrane and thus provides
 90 a means of intercellular communication. By forming a stable *AI – LuxR*
 91 complex, the transcription of a second copy of the repressilator gene *lacI* is
 92 activated. Placing the gene *luxI* under inhibitory control of the repressilator
 93 protein *TetR* (Fig. 1) introduces a rewiring between the repressilator and
 94 the quorum sensing through an additional loop, which competes with the
 95 overall negative feedback loop along the repressilator ring and results in an
 96 inhibitory, phase-repulsive intercellular coupling.

The *mRNA* dynamics is described by the following Hill-type kinetics with Hill coefficient n :

$$\dot{a}_i = -a_i + \frac{\alpha}{1 + C_i^n} \quad (1)$$

$$\dot{b}_i = -b_i + \frac{\alpha}{1 + A_i^n} \quad (2)$$

$$\dot{c}_i = -c_i + \frac{\alpha}{1 + B_i^n} + \kappa \frac{S_i}{1 + S_i} \quad (3)$$

where the subindex i denotes the cell ($i = 1, \dots, N$, N being the total number of cells in the ensemble), and a_i , b_i and c_i represent the concentrations of *mRNA* molecules transcribed from *tetR*, *cI* and *lacI*, respectively. The model is made dimensionless by measuring time in units of the *mRNA* lifetime (assumed equal for all genes) and the *mRNA* and protein levels in units of their Michaelis constants (assumed equal for all genes). The *mRNA* concentrations are additionally rescaled by the ratio of their protein degradation (different among the genes) and translation rates (assumed equal for all genes). After rescaling, α is the dimensionless transcription rate in the absence of a repressor, κ is the maximum transcription rate of the *LuxR* promoter, and the parameters $\beta_{a,b,c}$ describe the ratios between the *mRNA* and protein lifetimes (inverse degradation rates). We assume different lifetime ratios for the protein/*mRNA* pairs, which results in a weak relaxator-like dynamics of the repressilator (Ullner et al. (2007)). A_i , B_i , and C_i denote the concentration of the proteins *TetR*, *CI*, and *LacI*, whose dynamical behavior is given by:

$$\dot{A}_i = \beta_a(a_i - A_i) \quad (4)$$

$$\dot{B}_i = \beta_b(b_i - B_i) \quad (5)$$

$$\dot{C}_i = \beta_c(c_i - C_i) \quad (6)$$

Assuming equal lifetimes and dynamics for both the *CI* and *LuxI* proteins (since they are both repressed by TetR), we use the same variable to

describe the dynamics of both proteins. The *AI* concentration S_i in the i -th cell (rescaled additionally by its Michaelis constant) is proportional to B_i , i.e. the concentration of *LuxI* in it, and is further affected by an intracellular degradation and diffusion toward or from the intercellular space:

$$\dot{S}_i = -k_{s0}S_i + k_{s1}B_i - \eta(S_i - S_e) \quad (7)$$

$$S_e = Q\bar{S} \quad (8)$$

$$\bar{S} = \frac{1}{N} \sum_{i=1}^N S_i \quad (9)$$

97 The diffusion coefficient η depends on the permeability of the membrane
 98 to the autoinducer. Due to the fast diffusion of the extracellular *AI* (S_e)
 99 compared to the repressilator period, we can apply the quasi-steady-state
 100 approximation to the dynamics of the external *AI* and replace it by the mean
 101 field of the internal *AI*, \bar{S} . The parameter Q is defined as $Q = \frac{\delta N/V_{ext}}{k_{se} + \delta N/V_{ext}}$
 102 (García-Ojalvo et al. (2004)), with N being the total number of cells in
 103 the ensemble, V_{ext} the total extracellular volume, k_{se} the extracellular *AI*
 104 degradation rate, and δ the product of the membrane permeability and the
 105 surface area. In more general terms, Q is proportional to the cell density,
 106 and can be varied in a controlled way between 0 and 1 in experiment, thus
 107 making it a reasonable choice to follow the dynamical changes of the system
 108 with respect to Q .

109 Previous investigations carried on a minimal system of two repressilators
 110 coupled via repulsive cell-to-cell communication (Ullner et al. (2008)) have
 111 identified a variety of collective regimes, including constant level protein pro-
 112 duction (homogeneous steady state solution, HSS, Fig. 2a), an inhomoge-
 113 neous steady state characterized by different stationary protein levels (IHSS,

114 Fig. 2b), and self-sustained oscillations. Within the latter case, we have iden-
115 tified out-of-phase oscillations with different phase shifts (see e.g. Fig. 2c, for
116 which the phase shift is $\frac{\pi}{2}$), as well as a complex inhomogeneous limit cycle
117 (IHLC) characterized by one cell exhibiting very small oscillations around
118 a high mean protein level, whereas the second cell oscillates in the vicinity
119 of the steady state with an amplitude just slightly smaller than that of an
120 isolated oscillator (Fig. 2d).

121 The previous results correspond to a fixed value $N = 2$. However, *in*
122 *vivo* bacterial colonies proliferate and expand. In order to understand and
123 characterize the cooperative behavior in growing populations, it is certainly of
124 outmost significance to investigate the influence of the size of the population
125 on its dynamics. To that end, we performed a simple numerical experiment:
126 we computed 1000 time series with different random initial conditions for
127 a minimal ($N = 2$ cells), and a system with intermediate size ($N = 18$),
128 using a uniform distribution in the range $[0, 220]$ for the *mRNA* and protein
129 initial conditions and $[0, 1.2]$ for the *AI* initial conditions. Figure 3 shows
130 the histograms of detectable stable regimes in both systems for increasing
131 coupling coefficient Q .

132 As shown in Fig. 3, already a small increase in the system size (from
133 $N = 2$ to $N = 18$ cells) alters the balance between the coexisting regimes:
134 the stability regions of IHLC and IHSS are significantly increased at the
135 expense of the HSS. This fact underlines the connection between the size of
136 the population and its dynamical behavior, implying that a detailed analysis
137 of these correlations is necessary in order to reveal and formulate (predict) a
138 general statement about the cooperative behavior of growing populations.

139 3. Clustering in the inhomogeneous regimes

140 It is well known that genetically identical cells may exhibit diverse phe-
141 notypic states even under almost identical environmental conditions. Thus,
142 populations comprised of identical cellular units can display heterogeneity,
143 manifested by the existence of several subgroups or clusters where cells ex-
144 hibit organized collective behavior, with or without complex relations among
145 them. The size of the population plays a crucial role in determining which
146 dynamical behavior is most likely to be dominant, depending of course on
147 the environmental conditions as well as on the coupling strengths.

148 We now show that the parameter stability intervals for given solutions
149 increase significantly as a function of N , with respect to the equivalent stabil-
150 ity intervals in the minimal model of two coupled cells (Ullner et al. (2008)).
151 These changes in the dynamical structure of the model occur in general
152 due to the increased possibility for cluster formation in growing populations.
153 Clustering can be defined as a stable dynamical state characterized by the
154 coexistence of several subgroups where the oscillators exhibit identical (or
155 nearly identical) behavior. Clustering is a well known property, especially
156 for globally coupled systems, and has been investigated for identical phase
157 (Golomb et al. (1992); Okuda (1993)), salt-water (Miyakawa and Yamada
158 (2001)) or electrochemical oscillators (Wang et al. (2001); Kiss and Hudson
159 (2003)), in synthetic genetic networks (Koseska et al. (2007)), and in popu-
160 lations of chaotic oscillators (Kuznetsov and Kurths (2002); Manruiba and
161 Mikhailov (1999); Osipov et al. (2007)), among other cases. The presence of
162 clustering and the complex phase relations between cells produced therewith
163 can be very important in the construction of synthetic genetic networks and

164 the mechanisms behind cell differentiation. Therefore, our attention will be
165 mainly devoted to clustering that occurs in the inhomogeneous states (steady
166 or oscillatory), and which could be related to biological mechanisms of dy-
167 namical differentiation. Moreover, different groupings that occur mainly in
168 the chaotic regime and contribute significantly to the complex dynamical be-
169 havior on a population-wide scale will be also of significant interest to us.
170 This is because such groupings can be also seen as a mechanism for temporal
171 mixing that enhances the diversity in the system, while mostly maintaining
172 the advantages of a synchronized (ordered collective) behavior.

173 As a first step, a minimal extension to $N = 3$ identical cells was intro-
174 duced, in order to classify the dynamical changes leading to clustering. We
175 now present bifurcation diagrams for that case, with the coupling strength
176 Q as the bifurcation parameter. As discussed above, Q is proportional to the
177 extracellular cell density and can be changed experimentally in chemostat
178 experiments in the range between zero and one. Values beyond this range
179 do not have a biological meaning but can be helpful for the understanding
180 of the bifurcation analysis and the controlling of desired regimes. Although
181 the complete bifurcation analysis was performed using the *Xppaut* package
182 (Ermentrout (2002)), the diagrams presented here depict only those bifurca-
183 tion branches and points central to the corresponding discussion, in order to
184 avoid making the bifurcation charts incomprehensible.

185 The bifurcation analysis revealed a significant enlargement of the stability
186 interval for the *IHSS* ($Q \in [0.29 - 0.66]$, Fig. 4(b)), in comparison to the
187 minimal case of $N = 2$ coupled oscillators ($Q \in [0.36 - 0.55]$) (Fig. 4(a) and
188 in (Ullner et al. (2008))). The *IHSS* is stabilized via a Hopf bifurcation, thus

189 displaying no qualitative changes in the mechanism of occurrence with respect
 190 to the minimal model. However, the significant increase of the stability region
 191 in this case ($\approx 50\%$ in comparison to $N = 2$) is a result of clustering, or more
 192 specifically, of the increased number of possible distributions of the oscillators
 193 between the two stable protein levels through which the *IHSS* is defined. In
 194 general, given N total number of cells, the oscillators can have $N - 1$ different
 195 distributions between the clusters (considering that the *IHSS*, as well as the
 196 *IHLC* discussed below, are characterized by two-cluster decompositions).
 197 In what follows, we will define the different cluster states by the notation
 198 $mL | (N - m)U$, which denotes a cluster of m oscillators in the low-protein
 199 concentration state L , while the remaining $N - m$ oscillators populate the
 200 upper state U , characterized by higher protein concentration. For $N = 2$
 201 cells, there is only one possible distribution of the oscillators in the *IHSS*
 202 regime: $1L | 1U$ - one oscillator populates the lower, where the second one
 203 populates the upper state. However, for an increased number of cells, $N = 3$,
 204 there are 2 different combinations, namely $1L | 2U$ (Fig. 4b, left stable branch
 205 (solid (green) line)) and $2L | 1U$ (Fig. 4b, right stable branch (solid (green)
 206 line)). Note that different stable cluster distributions are located on separate
 207 branches of the bifurcation continuation, thus resulting in the increase of
 208 the parameter interval where *IHSS* exists. In the case of $N = 5$ oscillators,
 209 for example, 4 different clusters are stable, as shown in Fig. 4(c). In that
 210 diagram the cluster types are, from left to right: $1L | 4U$, $2L | 3U$, $3L | 2U$
 211 and $4L | 1U$. This particular structure of cluster distribution is typical for
 212 any number of oscillators: clusters of the type $1L | (N - 1)U$ require small Q
 213 values, while the $(N - 1)L | 1U$ exist for large Q . In order to understand this

214 behavior, let us define the ratio of the oscillators distributed in the upper
215 versus lower B_i states as $r = \frac{N-m}{m}$. Suppose that under small Q , r is larger
216 than one ($r > 1$ means that the majority of the oscillators in the system
217 are located in the upper B state). Let us now assume that the value of
218 r decreases, until $r < 1$. This means that the total concentration of the
219 protein CI (B) in the system is decreased. Moreover, since the dynamics of
220 the AI follows closely the dynamics of the protein B (both expressions, that
221 of protein B (cI) and of AI ($luxI$) are regulated in a same manner via $tetR$),
222 the production of internal AI will also be decreased. In order to compensate
223 for the lack of internal AI which will destabilize the $IHSS$, the re-influx of
224 external AI needs to be increased. This will subsequently lead to higher Q
225 values which means that in general, if $r < 1$, larger Q is necessary to observe
226 stable $IHSS$ distributions.

227 The left and middle plots in Fig. 5 show time traces for two different cluster
228 decomposition in the $IHSS$ regime for $N = 18$. Each possible partition
229 shows slightly different levels in the protein concentrations, and hence a fine
230 tuning of the protein levels can be accomplished by choosing a specific Q interval
231 for proper partition of the oscillators. This specific effect enhances, on
232 the one hand, the biotechnological applications of synthetic genetic networks
233 by providing a possible method for fine manipulation of the protein concentration
234 level, and on the other hand it might be seen as typical adaptability
235 of a cell population: when changes in the environmental conditions occur,
236 the population can easily adjust its cell distribution, adapting optimally to
237 the environment.

238 The Hopf bifurcation through which the $IHSS$ is stabilized gives rise also

239 to a branch of stable inhomogeneous limit cycles (*IHLC*), already introduced
 240 for the case of $N = 2$ oscillators in Fig. 2(d) (the corresponding bifurcation
 241 diagram is given in Fig. 6(a)). However, in comparison to that minimal case,
 242 the *IHLC* regime for higher population sizes is more complex, due to the
 243 increased number of possibilities for distributing the oscillators in the two
 244 clusters (a stable and an oscillating one).

245 Analogous to the formation of *IHSS* clusters for $N = 3$, two distinct
 246 *IHLC* clusters can be observed here as well. We have identified these regimes
 247 as $1L | 2U$ (left solid (red) line in Fig. 6, (b)), and $2L | 1U$ (right solid (red)
 248 line in Fig. 6 (b)), emerging from the correspondent *IHSS* branches (Hopf
 249 bifurcations of the *IHSS*, HB_{s1} and HB_{s2} in Figs. 4(b), 6(b)). This results
 250 again in an interval where the *IHLC* regime is stable in comparison to the
 251 2-oscillator case (compare Figs. 6 (a) and (b)). Moreover, the *IHSS* and
 252 *IHLC* regimes coexist in certain Q ranges (for instance, Figs. 5 and 6). This
 253 new behavior is also a result of the formation of clusters for increasing system
 254 size. The possibility that one of the *IHLC* distributions will overlap with the
 255 *IHSS* from another cluster distribution (containing less elements in the lower
 256 level) increases significantly. Hence, we can state that the increase of the
 257 stability regions of the inhomogeneous states (*IHSS* and *IHLC*) unraveled
 258 by the numerical simulations (Fig. 3) is a result of the cluster formation for
 259 growing populations, as we have determined from the bifurcation analysis
 260 presented.

261 Interestingly, the population displays even more complicated behavior
 262 when analysing the clustering effect in the *IHLC* regime. This complexity
 263 is manifested through the formation of sub-clusters in the lower (oscillatory)

264 state, where oscillators exhibit identical behavior within a single sub-cluster,
265 but with various phase relations among them. The simplest case consists of
266 only two oscillators located on the lower oscillatory level – they are organized
267 in anti-phase. However, increasing the number of oscillators in the lower state
268 reveals multitude of relations between the oscillators grouped in sub-clusters
269 and hence, besides the distribution of oscillators between the upper and lower
270 states, one needs to consider also the composition of the oscillatory sub-
271 clusters in the lower protein level. Figures 7 to 9 illustrate some examples of
272 possible combinations of partitions and phase relations in the sub-threshold
273 oscillations for an ensemble of $N = 18$ oscillators which we discuss next.

274 Due to the technical difficulty to handle a high-dimensional system with
275 the *Xppaut* package, we present here only numerical findings. Please note
276 the non-uniform distribution of the phase in some situations. The basic
277 distribution of IHLC can be seen in Fig. 7(left), with only one element in
278 the lower state. However, if an additional element is located in the lower
279 state as well, (Fig. 7, middle), the phase-repulsive cell-to-cell communication
280 evokes anti-phase oscillations, as already mentioned. In the situation where
281 three cells are located in the lower state, stable out-of-phase oscillations with
282 a phase-shift of $\frac{2\pi}{3}$ between clusters (Fig. 7, right) are observed, since the
283 phase-repulsive coupling maximizes their phase difference.

284 Additional oscillators in the lower state contribute to the formation of
285 more complex distributions. A fourth cell expressing a low *CI* protein con-
286 centration can establish a separate sub-cluster, which leads to the regime
287 $(1 : 1 : 1 : 1)L | 14U$ (each sub-cluster is composed of isolated cells, as shown
288 in Figs. 8, left and middle) or to a sub-cluster with more than one cell (e.g.

289 two sub-clusters are formed, each containing two cells, as in Fig. 9 middle).
290 It is interesting to note, that the regime $(1 : 1 : 1 : 1)L \mid 14U$ has two re-
291 alizations with different phase relations between the self-oscillatory cells in
292 the low CI level. The left panel of Fig. 8 shows a nearly equal spaced phase
293 shift of about $\frac{2\pi}{4}$, while the middle panel shows the case of an inhomogeneous
294 phase shift. In particular, the cells are observed to come close to each other
295 (two of them), but they never merge to form a sub-cluster. The right panel
296 in Fig. 8 shows a more complex phase relation in which 5 out of the 18 cells
297 are in the low-protein state, oscillating separately with an inhomogeneous
298 phase-difference. This phase-regime is similar to the one in the middle plot
299 of Fig. 8, but with an additional oscillator in between the other four clusters.
300 The corresponding six time series given in Figs. 7–8 correspond to the same
301 parameter values, for $N = 18$ and a fixed coupling value $Q = 0.2$. Thus
302 the different dynamical behavior observed here originates only from different
303 initial conditions.

304 It is important to note once again that a particular cluster distribution
305 is characterized with a distinct level of protein concentration expressed in
306 the cell. Namely, larger number of oscillators in the lower state reduces the
307 protein concentration in the higher state. Moreover, we have also observed
308 that the ratio ($r = \frac{N-m}{m}$) of the number of oscillators in both levels affects
309 the amplitude of the limit cycle oscillations located in the lower protein level:
310 $r < 1$ results in increased amplitude values. Additionally, the ratio r con-
311 tributes to the changes in the period of oscillation, having as a consequence
312 a well pronounced multirhythmicity in the system. Table 1 lists the observed
313 periods and phase relations for the different ratios in the cases discussed in

314 Figs. 7–8. In the present case of $N = 18$ and $Q = 0.2$, every additional
315 oscillator in the lower CI level of the IHLC lengthens the period by ≈ 1.2
316 time units, which leads to a significant change in the period between different
317 distributions. A modification of the phase relation by a fixed partition ratio
318 does not influence the period (left and middle panels of Fig. 8).

319 Due to the dynamical complexity of the system, which as mentioned
320 above is a direct consequence of the clustering, it is useful to look into the
321 stability of the different regimes (HSS , $IHSS$, $IHLC$), in order to define
322 a general prediction scheme to determine which solution is dominant under
323 different conditions. We have therefore calculated 1000 time series for a
324 growing population size N , with different random initial conditions for every
325 parameter set, using the approach discussed in Sec. 2. These initial conditions
326 cover the $7N$ -dimensional phase space of the system (7 degrees of freedom
327 per oscillator) densely enough such that one can detect stable coexisting
328 attractors with a significant basin of attraction. Figure 10 shows in detail the
329 system size effect for two specific coupling values, ($Q = 0.24$ and $Q = 0.3$).
330 In both cases, the results show a clearly monotonic increase of the probability
331 that the $IHSS$ is reached from random initial conditions at the expense of the
332 HSS , as the size of the population grows. For ensembles larger than several
333 hundred cells the $IHSS$ is the dominant region, allowing us to speculate that
334 the artificial differentiation of cells is strongly dependent on the size of the
335 population, becoming more likely with proliferation (Koseska et al. (2009)).
336 In other words, the stable inhomogeneous steady state resembles Turing’s
337 dissipative structure (Turing (1952)), only without space variables. In a
338 sense, instead of the spatial Turing structure, in $IHSS$ we have a two cluster

339 decomposition present. This state, as discussed above, is characterized by
340 two different stable protein concentration levels, which might be biologically
341 interpreted as dynamical differentiation. Thus, one can further speculate
342 that a robust dynamical differentiation of the cells strongly depends on the
343 size of the population.

344 **4. Full-amplitude oscillatory regimes - Regular and chaotic attrac-** 345 **tors**

346 For couplings smaller than a given critical value $Q_{\text{crit}} \sim 0.129$ (the value
347 of Q_{crit} slightly varies depending on N), the system can only exhibit self-
348 oscillatory solutions: we have identified out-of-phase oscillations with a num-
349 ber of different phase-shifts (e.g. $\frac{\pi}{2}$, $\frac{3\pi}{4}$, etc.). In contrast to the *IHLC* so-
350 lution, the attractors here share the same phase space. These full-amplitude
351 oscillatory regimes, as we will further denote them, are dynamically very
352 rich, displaying a diversity of sub-regimes for increasing coupling values. In
353 particular, similarly to the minimal system of $N = 2$, we found two main
354 types of behavior depending on the coupling strength Q :

- 355 • regular oscillations with stable cluster formation;
- 356 • chaotic self-oscillations with only a temporary cluster formation (we
357 will denote this as grouping in the following discussion).

358 In what follows, we investigate the general characteristics of these regimes
359 by means of bifurcation and numerical analysis. However, in order to dis-
360 tinguish between separate cluster formations for increasing population sizes
361 via numerical simulations, we use the following definition: oscillators i and

362 j belong to the same cluster K at time t if the difference between the inter-
 363 nal AI concentrations $S_i(t)$ and $S_j(t)$ at consecutive sampling time events is
 364 smaller than a pre-defined value $\varepsilon = 0.001$:

$$\begin{aligned} & [\text{osc}_i(t), \text{osc}_j(t)] \in \text{clust}_K(t) \\ & \text{if } |S_i(t) - S_j(t)| \leq \varepsilon \text{ and} \\ & |S_i(t - \Delta t) - S_j(t - \Delta t)| \leq \varepsilon . \end{aligned} \quad (10)$$

365 The cluster sampling occurs every $\Delta t = 64$ time units, which is larger than
 366 the average period of the oscillations (the average period of oscillations is
 367 between 40 and 50 time units or approx. 200 min). Using these criteria, we
 368 classify the clustering of the oscillators, and present the resulting structures
 369 in the form of cluster plots (Figs. 12, 13, 15, 16), where the x -axes denote
 370 time, and the y -axes represent the oscillator index. There, we use different
 371 colors to encode the cluster number to which each element belongs (white
 372 color represents a free-running oscillator, which does not belong to any of
 373 the clusters). Although these plots do not show the dynamics of the self-
 374 oscillations in detail, they focus on the difference in the protein concentrations
 375 of separate cells over time, and therefore enable a visualization of the long-
 376 time cluster dynamics.

377 4.1. Regular attractors

378 Under small and intermediate couplings ($0 \leq Q \lesssim 0.55$), the system
 379 demonstrates regular oscillations with a fixed unique amplitude and com-
 380 mon period for all oscillators (Fig. 12, top panel). In this case, the repulsive
 381 cell-to-cell communication evokes the preference of phase-shifted oscillations,

382 and small ensembles with $N \leq 4$ show solutions without clustering and ho-
 383 mogeneously distributed phases (Fig. 2c). The phase shift here depends on
 384 the size of the system, and obeys the relation $\frac{2\pi}{N}$ (a solution known also
 385 as a "splay-phase" solution (Kaneko (1991); Watanabe and Strogatz (1993);
 386 Nicolis and Wiesenfeld (1992))). Hence, in a system of three coupled repres-
 387 silators, one can find stable full-amplitude oscillations phase-shifted by $\frac{2\pi}{3}$
 388 between HB_1 ($Q = 1.253$) and TR_1 (torus bifurcation for $Q = 1.11$), and
 389 from $Q = 0$ until $\text{TR}_2 = 0.55$ (see Fig. 11). No other stable cluster decom-
 390 position was identified from the bifurcation analysis. For $Q < 0.129$, and as
 391 mentioned above, this is the only stable solution of the system, whereas for
 392 increasing coupling the full-amplitude regime coexists with HSS, IHSS, and
 393 IHLC states, as shown in Fig. 11.

394 In systems where $N > 5$, clustering is observed in the regular oscillatory
 395 regime. Here, the 3-cluster decomposition dominates, with a nearly equal
 396 number of oscillators in each one (for details see Table 2), and a distinct phase
 397 relation between separate clusters. Moreover, the periods of oscillation are
 398 slightly shorter than those of isolated elements, and decrease as Q increases
 399 (Table 2). We show here an example of a system of $N = 18$ cells. The
 400 onset of clustering can be seen in the cluster plot in the bottom panel of
 401 Fig. 12. After a long transient (about 1.200 time units, in this particular
 402 case) a distribution of three clusters is stabilized, with a $7 : 6 : 5$ distribution
 403 of oscillators (cells) between the clusters, and a phase shift of about $\sim \frac{2\pi}{3}$
 404 among them. Time series of the separate clusters (the oscillators within
 405 each cluster display synchronous behavior) are given in the top panel of
 406 Fig. 12 (the coloring corresponds to the cluster plot). The long transient in

407 the simulation looks unphysiological at first glance, but all simulations are
408 drawn from random initial conditions with a very large diversity amongst
409 the cells. We use those unrealistic initial conditions in order to underline
410 the ability of the system to form stable clusters under any condition. After
411 proliferation, the daughter cells are in a similar phase as the mother, which
412 decreases the formation of stable clusters significantly.

413 In the cases investigated, all the oscillators are identical and coupled via a
414 mean field, hence there are no preferences amongst them to establish a given
415 set of clusters. Thus the distribution of the oscillators between the clusters
416 depends exclusively on the initial conditions. Several typical attractors ob-
417 served for different system sizes and coupling values Q are listed in Table
418 2, together with the values of their periods. As shown here, the 3-cluster
419 decomposition with nearly equal distribution of oscillators between the clus-
420 ters dominates for large system sizes and over wide ranges of coupling. An
421 exception to this case, as discussed above, is the formation of clusters for
422 $N \leq 4$. In the case for $N = 4$, for instance, the 3-cluster decompositions
423 lose stability, and stable 4-cluster decompositions are formed. Interestingly,
424 the coupling Q has an inverse influence on the oscillation period. Normally,
425 stronger coupling lengthens the period of coupled systems (Crowley and Ep-
426 stein (1989); Volkov and Stolyarov (1994)), but the situation is different in
427 the present case because Q controls the reinflux of AI and a higher internal
428 AI concentration shortens the repressilator cycle. Compared to the coupling
429 strength Q , the system size N and the cluster composition have a minor
430 influence on the period.

431 *4.2. Influence of parameter heterogeneity on the regular-attractor regime*

432 The previous investigations analyzed in detail the dynamics of the regu-
433 lar attractors, as a first step in the understanding of the global cooperative
434 behavior of large populations. However, the assumption that the elements
435 of the system are identical (differing only in the initial conditions) is very
436 strong, since cellular populations are heterogenous. It is therefore important
437 to account for diversity among parameter values in separate cells by introduc-
438 ing, for e.g. certain mismatch in the α parameter values. In particular, we
439 consider here that for each cell $i = 1, \dots, 11$ different α 's are assigned from
440 a defined set of values $[210, 211, \dots, 220]$, which leads to variability larger
441 than 3% in the oscillation periods. Introducing the diversity exactly in α
442 is realistic, since this parameter defines the expression strength of the re-
443 pressor genes, which is proportional to the concentration of repressor
444 plasmids present in the cell. The control of the number of plasmid copies in
445 experiments was discussed in (Paulsson and Ehrenberg (2001)), and it can
446 be coordinated with the cell's growth and division. Additionally, an increase
447 in α lengthens the period of oscillations, as already mentioned.

448 We have observed that even in the presence of diversity, the three-cluster
449 decomposition is the most probable state in the system (an example for
450 $Q = 0.5$ is given in Fig. 13). However, in contrast to the case of identical
451 oscillators, some of the cells (in the case of Fig. 13, the two elements with
452 the smallest parameter α , i.e. the cells with the shortest period), are not
453 phase locked and jump periodically from one of the three stable clusters to
454 the other one. Moreover, the heterogeneity introduced via the parameter
455 mismatch breaks the symmetry present in the system of identical oscillators,

456 and leads to a situation where oscillators with similar properties (i.e. similar
457 α_i) group together in a cluster, preferentially.

458 4.3. Irregular self-oscillations

459 The bifurcation analysis we have performed on the model for $N = 3$ cells
460 shows that for $Q \gtrsim 0.55$, the system goes beyond the range of regular oscil-
461 lations: the periodic branch loses its stability between two torus bifurcations
462 (TR_1 and TR_2 in Fig. 11), which contributes to the appearance of oscilla-
463 tions with strong variations of the amplitude. These irregular oscillations
464 look very similar to chaotic time series (Fig. 15, top plot), however, in order
465 to classify this as a chaotic behavior, certain criteria need to be fulfilled,
466 e.g. at least one of the Lyapunov exponents of the system needs to be posi-
467 tive. Therefore, we integrate forward in time a small perturbation of the
468 trajectory, the random tangent vector, by means of the Jacobi matrix. The
469 logarithm of the norm of the tangent vector is related to the maximal Lya-
470 punov exponent (Eckmann and Ruelle (1985)) and we normalize it by the
471 integration time. The result is plotted in the top panel of Fig. 14, and shows
472 that for $Q > Q_{chaos} \approx 0.6$ (upper boundary of $Q \approx 1$), clear chaotic behavior
473 with a positive maximal Lyapunov exponent is observed. The bottom panel
474 of Fig. 14 displays a bifurcation diagram computed as a series of Poincaré
475 sections, with the ordinate showing the value of the B_1 when the trajectory
476 crosses $A_1 = 4.0$. We avoid the tracking and evaluation of unstable attractors
477 and numerical artifacts by adding small dynamical noise to the transcription
478 dynamics of the *tetR mRNA*, i.e. we add the term $\xi_i(t)$ to the rhs of Eq. 1.
479 The local noise term $\xi_i(t)$ is assumed to be Gaussian, with zero mean and
480 intensity σ_a^2 defined by the correlation $\langle \xi_i(t)\xi_j(t + \tau) \rangle = \sigma_a^2 \delta(\tau)\delta_{i,j}$. Beside

481 being technically useful, the noise is biologically relevant, as it is caused by
482 random fluctuations in transcription due to the small number of involved
483 *mRNA* (Elowitz and Leibler (2000)).

484 In contrast to the case of the minimal system ($N = 2$), where a similar
485 irregular dynamic with chaotic behavior was observed (Ullner et al. (2008)),
486 the extended system studied here ($N = 18$ in Fig. 14) is characterized by
487 the ability to build temporal clusters, which we refer to as grouping. The
488 coupling Q changes the chaotic behavior gradually (top panel in Fig. 14).
489 A first weak increase of the maximal Lyapunov exponent is followed by a
490 fast rising interrupted by short collapse and a final decline to zero at $Q \approx 1$.
491 The degree of chaos influences the grouping ability, as chaos destabilizes and
492 shortens the grouping. In the parameter range of Q where more irregular than
493 simple periodic oscillations are observed, $0.55 \lesssim Q$, temporal 2-, 3-, 4- or
494 5- grouping decompositions with a significant lifetime have been observed,
495 in contrast to the regular attractors, where the 3-cluster decompositions
496 were the dominant ones.

497 The example in Fig. 15 shows a weak chaotic dynamics of $N = 18$ os-
498 cillators at $Q = 0.6$, with long-living 3- and 4-grouping constellations. The
499 cluster plot (bottom panel of the figure) illustrates the interplay of long-time
500 grouping and recurring transients with less ordered states, while a rearrange-
501 ment to a new grouping happens. The groupings are long living up to 20,000
502 time units, i.e. about 4000 cycles. Due to the symmetry of the system in
503 the case of identical elements, separate oscillators do not have local group-
504 ing preferences. The bottom plot of Fig. 15 gives a detailed insight on the
505 oscillatory dynamics at different times, and shows the long-living 3- and 4-

506 grouping constellations, and transients with a high degree of decomposition.
507 However, once the oscillators are distributed in a long-living grouping state,
508 they oscillate synchronously within the group and cannot be distinguished by
509 their time series until the next decomposition occurs and spreads the phases.

510 The second example of irregular chaotic self-oscillations (Fig. 16) illus-
511 trates a regime of fully developed chaos at high coupling $Q = 0.75$. The max-
512 imal Lyapunov exponent (Fig. 14) increases significantly above zero, which
513 confirms the chaotic character of the dynamics. Interestingly, the temporal
514 grouping of the oscillators is conserved, but with a significantly shorter life-
515 time and faster mixing as compared to the weak chaotic dynamics discussed
516 above (Fig. 15). Despite the fact that clusters are not stable in this param-
517 eter range, some of the oscillators run in-phase over long time and fulfill the
518 clustering condition (Eq. 10) temporarily. In this typical situation shown
519 here for fully developed chaos at $Q = 0.75$ (Fig. 16) the grouping of the
520 oscillators can last over a relatively long time and cover up to 5,000 time
521 units, i.e. more than 100 oscillations. The individual repressilators oscillate
522 in an irregular manner with fluctuating amplitude and period. The top pan-
523 els of Fig. 16 shows different snapshots of the time series, and the bottom
524 panel of the figure shows the corresponding cluster plot and illustrates the
525 interplay between grouping and decomposition. Note the larger maximal am-
526 plitudes in the more ordered case than in the situation where higher degree
527 of decomposition is observed.

528 In general, it can be stated that inside the chaotic ensemble there exists
529 an everlasting tendency to build and break temporal groups, which leads to
530 their mixing. Many different temporal distributions of the oscillators into

531 groups are possible, which survive over several oscillation periods until the
532 next mixing occurs. In the case investigated here in detail, $N = 18$ oscilla-
533 tors, we have observed e.g. two temporal groups with a distribution $9 : 9$ of
534 the oscillators between them, three groups with distributions $7 : 6 : 5$, and
535 $7 : 7 : 4$, four temporal groups with distributions $8 : 6 : 3 : 1$, $7 : 6 : 4 : 1$,
536 $5 : 5 : 5 : 3$, and $5 : 5 : 4 : 4$, as well as several examples of five groupings
537 decompositions, with distributions such as $6 : 5 : 4 : 2 : 1$, $5 : 5 : 4 : 2 : 2$,
538 $5 : 5 : 4 : 3 : 1$ or $5 : 4 : 4 : 3 : 2$. Several other examples with a
539 higher degree of decomposition can be observed as well, however, they ex-
540 hibit a much shorter lifetime. The observed clustering effects resemble the
541 dynamical behavior of globally coupled maps reported by Kaneko in (Kaneko
542 (1990)). The transition from an ordered to a partially ordered and turbulent
543 phase, where the number of clusters is significantly increased is similar to
544 the case of a weak and well developed chaotic clustering decomposition or-
545 dering, discussed in this section. Moreover, we show that a growing system
546 size increases the possibility for grouping formation significantly, as well as
547 the number of different distributions of the oscillators between the groups,
548 which on the other hand enhances the flexibility of the system. This means
549 that by varying environmental conditions, the population can switch between
550 different distributions to adapt to its surroundings and improve its fitness.
551 Although the chaotic dynamics observed here and the effect of intrinsic noise
552 in synthetic oscillators (Elowitz and Leibler (2000); Stricker et al. (2008))
553 have very similar manifestations despite their different origins, we intend to
554 draw the readers' attention to chaos as an alternative source of uncertainty
555 in genetic networks.

556 The chaotic dynamics and the grouping phenomena appear gradually
557 for increasing coupling Q , i.e. at cell densities which could be a cause for
558 stress. One could speculate, that the population has the flexibility to respond
559 to environmental stress by distributing its cells within stable clusters, and
560 thus increasing the variability and diversity amongst the different cells to
561 enhance the probability to survive the stress situation. The gradual chaotic
562 behavior enables the population to adapt the mixing velocity and the degree
563 of diversity to the stress conditions.

564 5. Discussion

565 The mechanism how a multicellular system assures a robust and coordi-
566 nated response in a noisy and fluctuating environment is still an intriguing
567 question. It has been suggested however, that the intercellular signaling plays
568 one of the crucial roles in the establishment of cooperative functioning in
569 populations. In that context, we attempt here to characterize the dynamical
570 behavior of multicellular systems using phase-repulsively coupled synthetic
571 genetic repressilators [Eqs. (1)–(7)]. The focus in the current paper is on the
572 dynamics of large and growing ensembles, but we also compare our results
573 with the recent findings on the basic ensemble of two coupled repressilators,
574 by means of numerical simulations of the dynamics and bifurcation analysis.

575 We show that a multicellular population of synthetic genetic repressila-
576 tors displays various dynamical behavior, e.g. full-amplitude self-oscillations,
577 homogeneous steady state (HSS), inhomogeneous steady state ($IHSS$), and
578 inhomogeneous limit cycle ($IHLC$). These regimes are present for all popu-
579 lation sizes, and may in general coexist with each other. Moreover, the size of

580 the system affects the relative sizes of the basins of attraction of each regime.
581 For instance, the inhomogeneous states become more likely for larger popu-
582 lations. Interestingly, those inhomogeneous regimes can be associated with
583 permanent artificial cell differentiation in synthetic genetic networks, and
584 the simulations predict that a growing system size, e.g. due to proliferation,
585 enhances the probability of differentiation. Additionally, large system sizes
586 widen the parameter range of the *IHLC* and *IHSS* regimes significantly,
587 and further enhance the differentiation probability.

588 Furthermore, the understanding of cell differentiation (Kaneko and Yomo
589 (1997); Furusawa and Kaneko (2001)) and its connection to the emergence
590 of stable attractors from cell-to-cell coupling is still not clear. Here, we
591 have investigated systematically the mechanisms through which coexisting
592 attractors arise in the multicellular system. Namely, a closer look into the
593 inhomogeneous regimes (*IHLC* and *IHSS*) shows a splitting of the single at-
594 tractor that exists for two coupled elements into multiple coexisting solutions
595 for many oscillators, and the number of stable attractors increases with the
596 system size. A combination of numerical simulations and bifurcation analysis
597 revealed that the different stable *IHSS* solution branches differ by the num-
598 ber of elements in the high and low protein levels, and that each distribution
599 implies a new attractor with different stability ranges and individual protein
600 levels. The *IHLC* is on the other hand, directly bounded to the *IHSS* with
601 the same distribution of the oscillators via a Hopf bifurcation. The ability of
602 the *IHLC* regime to build oscillating clusters with different phase relations
603 in the low protein level increases further the number of sub-regimes, and in-
604 cludes multi-rhythmicity as a tunable clock (similarly to the tunability of the

605 *IHSS* regimes) because the attractors express different typical frequencies.
606 One can further speculate that this feature can be seen as an example of
607 the adaptability of a population to easily adjust its cell distribution when
608 environmental changes occur, in order to respond and adapt optimally to the
609 surrounding.

610 Similar behavior was observed in the full-amplitude self-oscillatory regime:
611 various oscillatory clusters with different partitions of the oscillators amongst
612 them. Moreover, the spectra of possible constellations increases with the sys-
613 tem size. For small and intermediate coupling Q , stable self-oscillations char-
614 acterized with a 3-cluster decomposition, and phase shifted by $\sim \frac{2\pi}{3}$ appear.
615 These 3-cluster decompositions are very robust to perturbations, in the form
616 of e.g. random initial conditions, dynamical noise or parameter heterogene-
617 ity. The direct numerical investigations performed here can not guarantee
618 the "mathematical" stability of the discussed clustering regimes. However,
619 taking in mind recent results (Ashwin et al. (2007)) we can suggest that these
620 clusters belong to a heteroclinic network and demonstrate switching which is
621 manifested more effectively in the presence of detuning. This question itself
622 deserves further attention but in any case, the regimes observed here have
623 very long life times which certainly makes them interesting and important
624 for biology.

625 Furthermore, an increasing cell density caused by cell growth and pro-
626 liferation increases effectively the coupling Q , and up to a critical coupling
627 $Q_{\text{crit}} \approx 0.6$ the regular self-oscillations become unstable, turning into chaotic
628 oscillations with high variability in their amplitude and frequency. Interest-
629 ingly, also in the presence of chaos the population tends to build temporal

630 clusters, which we refer to as groups. These temporal groups of co-jointly
631 oscillating repressilators have a significant lifetime, depending on the degree
632 of chaos, and are interrupted by recurring decomposition of the groups and
633 a reassembling into different groups. The chaotic dynamics appears gradu-
634 ally with Q , and allows the population to respond flexibly and sensitively to
635 increasing stress via a higher dynamical diversity inside the ensemble.

636 In summary, our results show that a population of synthetic genetic clocks
637 coupled via the mean field exhibit a significantly enhanced range of possi-
638 ble dynamical regimes with very different properties. One could speculate
639 that the observed multi-stability and multi-rhythmicity, which increase with
640 the system size, enhance the fitness of the cellular population under envi-
641 ronmental stress, and optimize the adaptation of the colony by a sensitive
642 adjustment of the protein dynamics.

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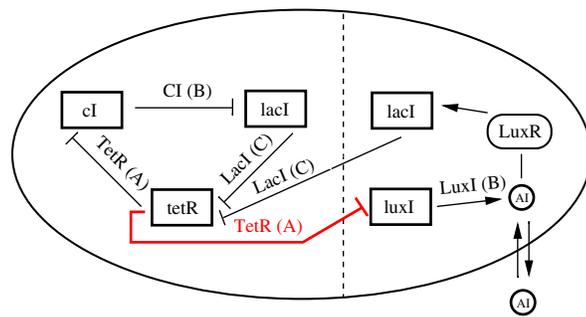


Figure 1:

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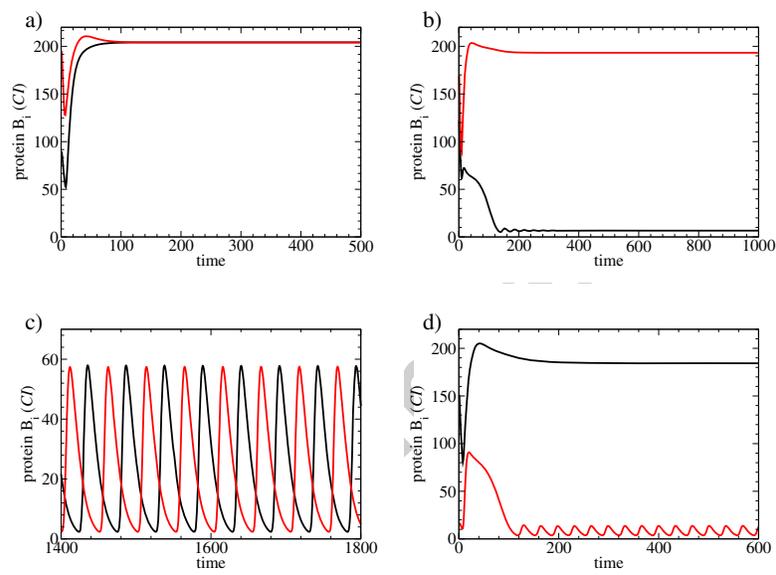


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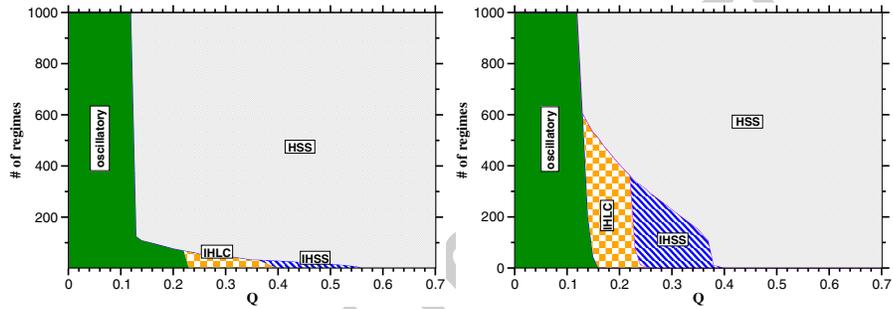


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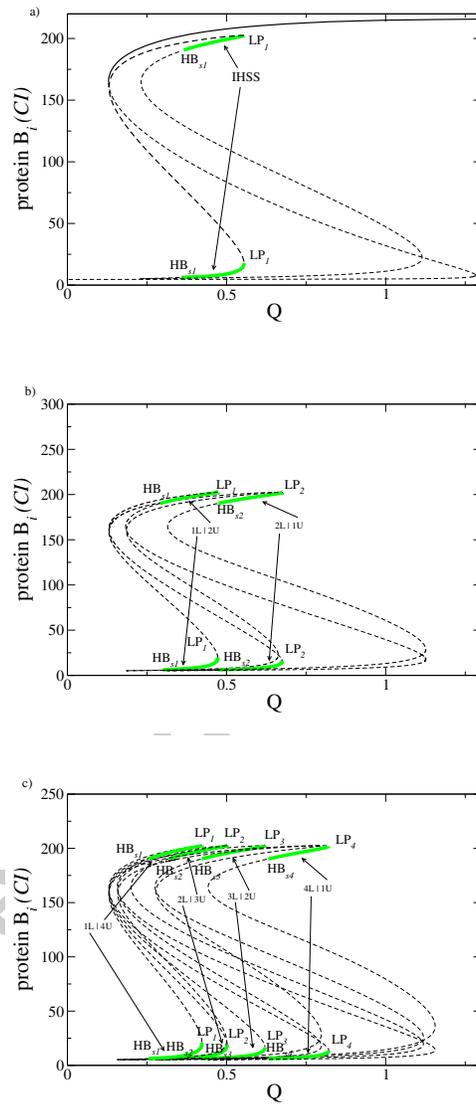


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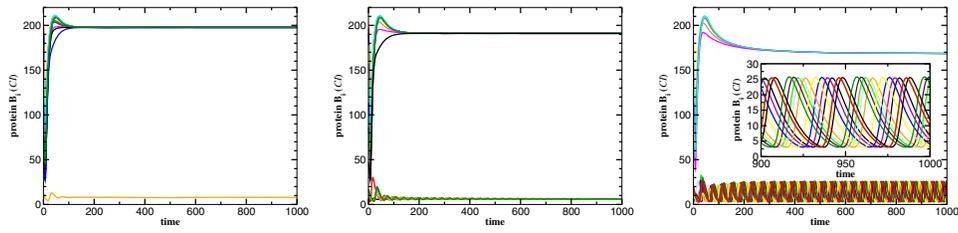


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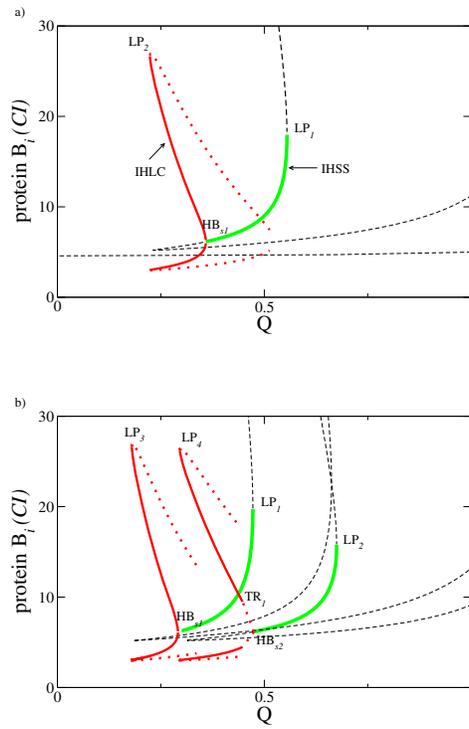


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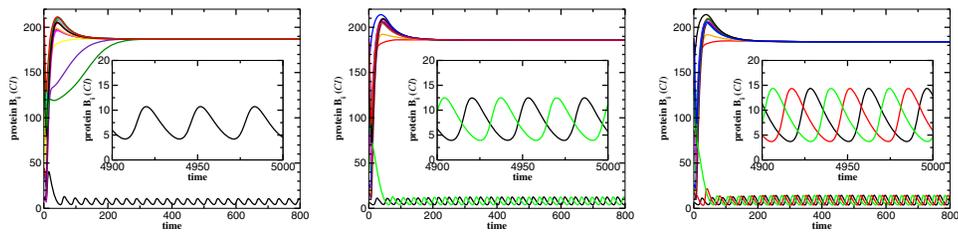


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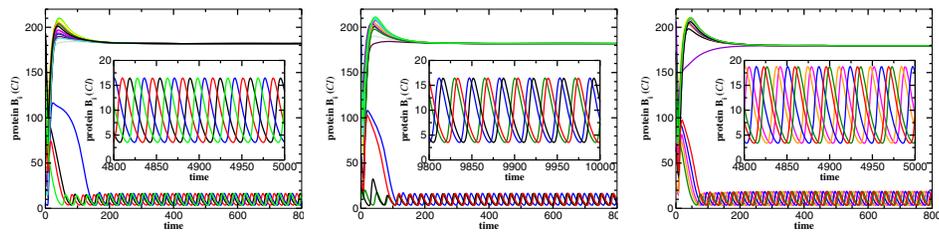


Figure 8:

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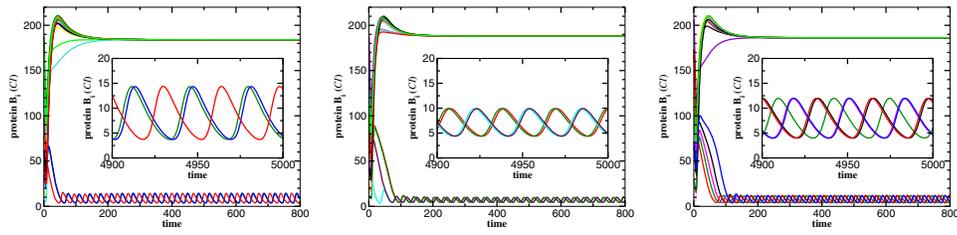


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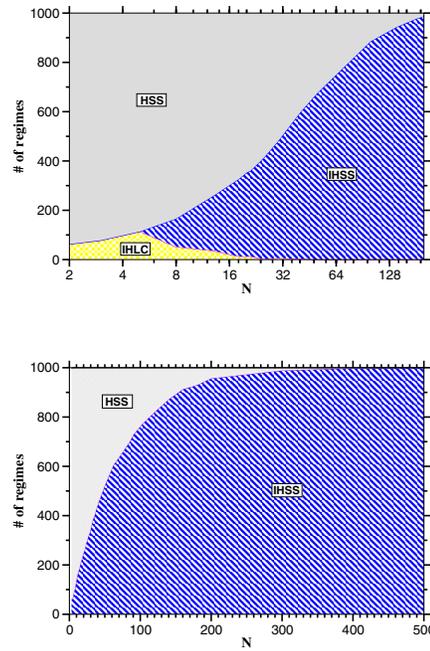


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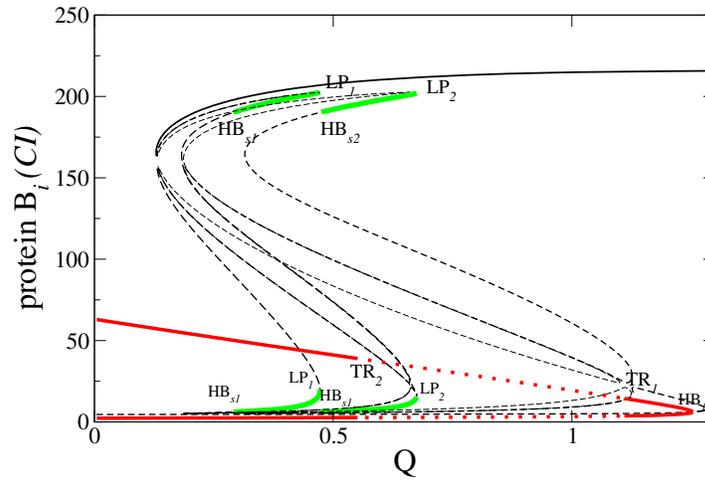


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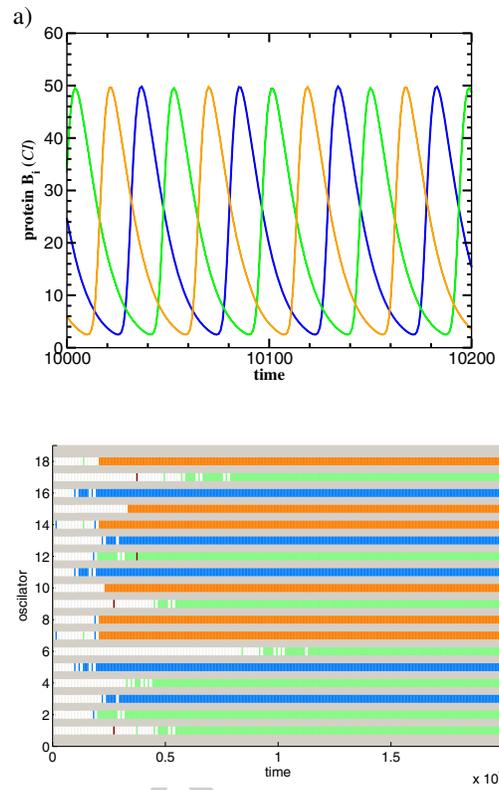


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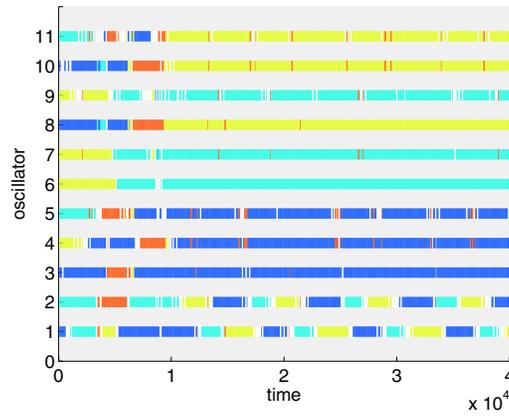


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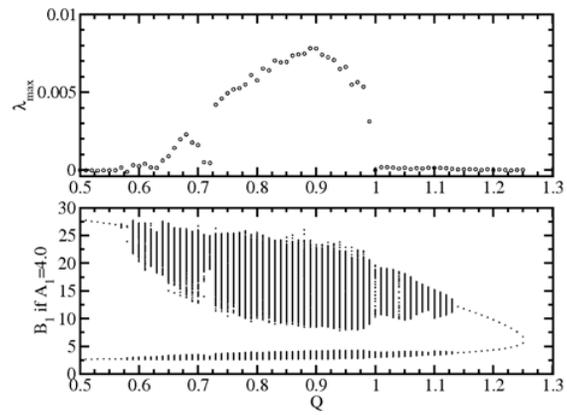


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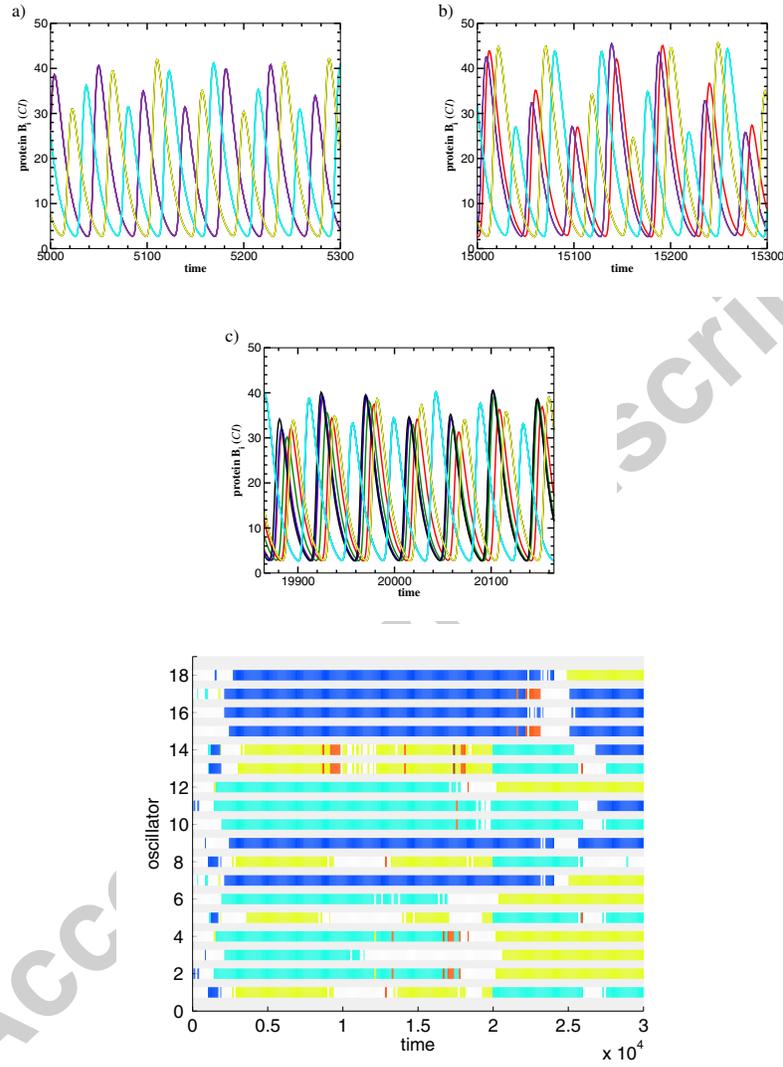


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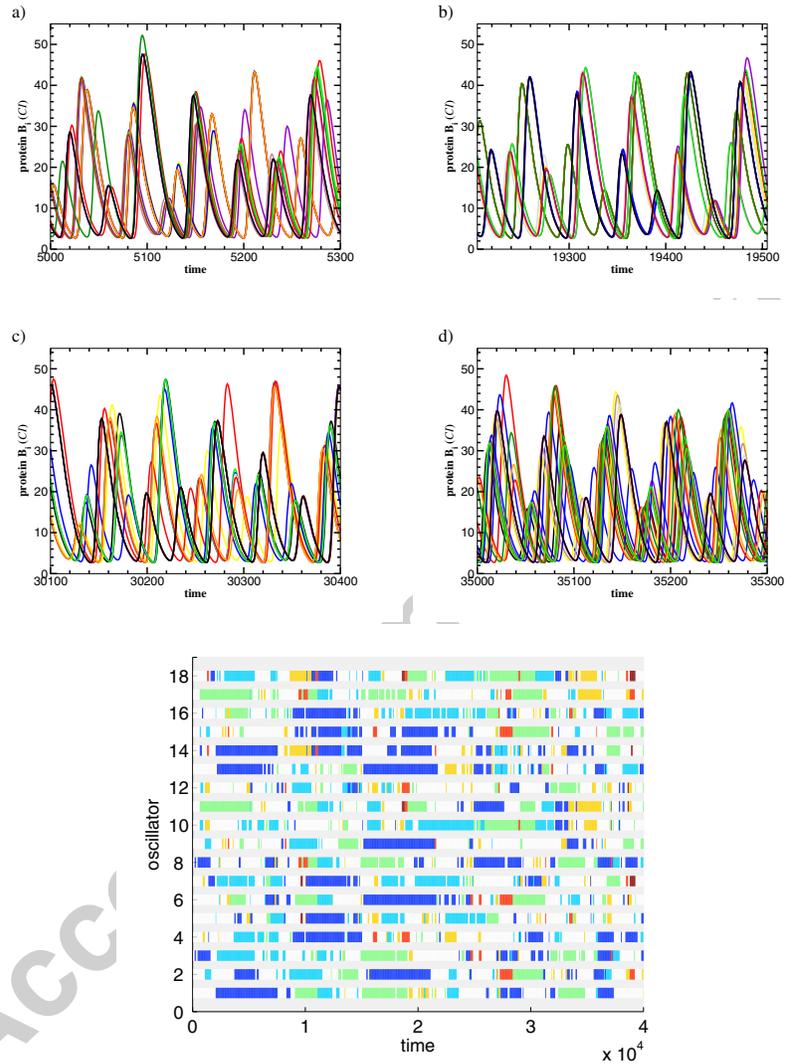


Figure 16:

747 **Figure Legends**748 *Figure 1.*

749 **Scheme of the repressilator with repulsive quorum sensing cell-**
750 **to-cell communication.**

751 *Figure 2.*

752 **Time series for different dynamical regimes for the minimal cou-**
753 **pled system of $N = 2$: a) $Q = 0.4$, homogeneous steady state; b)**
754 **$Q = 0.4$, inhomogeneous steady state; c) $Q = 0.1$, full amplitude**
755 **oscillations and d) $Q = 0.3$, inhomogeneous limit cycle. The other**
756 **parameters are: $n = 2.6$, $\alpha = 216$, $\beta_a = 0.85$, $\beta_b = 0.1$, $\beta_c = 0.1$, $\kappa = 25$,**
757 **$k_{s0} = 1.0$, $k_{s1} = 0.01$, $\eta = 2.0$.**

758 *Figure 3.*

759 **Influence of the system size ($N = 2$ in the left plot and $N = 18$**
760 **in the right plot) on the relative regime separation versus coupling**
761 **strength Q . Other parameters as in Fig. 2.**

762 *Figure 4.*

763 **Bifurcation chart depicting stable IHSS for: (a) $N = 2$; (b) $N = 3$**
764 **and (c) $N = 5$ oscillators. Other parameters as in Fig. 2. Here, thick**
765 **(green) solid lines denote stable *IHSS* cluster decompositions, and**
766 **dashed lines denote unstable steady state. Note that the bifurca-**
767 **tion charts are not complete, depicting only those parts relevant**
768 **for the current discussion.**

769 *Figure 5.*

770 Time series of protein CI (B_i) for the IHSS in two different
 771 cluster distributions: $1L | U17$ (left plot) and $6L | 12U$ (middle
 772 plot) and an example of IHLC (right plot) coexisting for the same
 773 parameters, with fixed system size $N = 18$ and $Q = 0.3$. The IHLC
 774 example contains 6 oscillators in the upper CI state and 12 in the
 775 lower one.

776 *Figure 6.*

777 Bifurcation structure of the IHLC regime for (a) $N = 2$, solid
 778 (red) line, and (b) $N = 3$ oscillators - the left solid (red) line denotes
 779 stable $1L | 2U$ distribution, whereas the right one denotes stable $2L |$
 780 $1U$ distribution. Other parameters as in Fig. 2. Due to the large
 781 stiffness of our multidimensional model and the proximity to the
 782 bifurcation point, the correct continuation could not be performed
 783 with the *Xppaut* package. Therefore, the bifurcation branches on
 784 this figure are not closed.

785 *Figure 7.*

786 Examples of different IHLC distributions in an ensemble of $N =$
 787 18 cells for the same coupling $Q = 0.2$. Every oscillatory sub-cluster
 788 consists of only one cell: $1L | 17U$ (left), $1 : 1L | 16U$ (middle), and
 789 $1 : 1 : 1L | 15U$ (right). The insets show a detail of the low-level
 790 oscillations after transients. Other parameters as in Fig. 2.

791 *Figure 8.*

792 *IHLC* states exhibiting oscillatory low-protein-level sub-regimes
 793 with four (left and middle) and five (right) elements in an ensemble
 794 of $N = 18$ cells. Every sub-cluster in the oscillatory state consists of
 795 only one cell. Here $Q = 0.2$ and other parameters as in Fig. 2. The
 796 left figure depicts the situation with equal phase distance between
 797 the oscillating repressilators in the low protein B level, while in the
 798 middle and right plots the phase distances are different. The insets
 799 belong to the same time series and show a detail of the oscillatory
 800 sub-clusters.

801 *Figure 9.*

802 Examples of IHLC states with complex phase relations between
 803 the oscillatory sub-clusters in an ensemble of $N = 18$ cells. The
 804 insets show in detail the small limit cycle oscillations in the lower-
 805 protein state. Parameters are $Q = 0.2$ with $(2 : 1)L | 15U$ (left),
 806 $Q = 0.24$ with $(2 : 2)L | 14U$ (middle), $Q = 0.24$ with $(2 : 2 : 1)L | 13U$
 807 (right). Other parameters as in Fig. 2.

808 *Figure 10.*

809 Distribution of dynamical regimes (HSS, IHSS, IHLC) for in-
 810 creasing cell numbers. The coupling strength is fixed to $Q = 0.24$
 811 (top) and $Q = 0.3$ (bottom). Other parameters as in Fig. 2.

812 *Figure 11.*

813 Bifurcation diagram of 3 coupled repressilators for increasing
 814 Q , illustrating the stability of different steady-state and periodic

815 branches. Due to limitations of the *Xppaut* package to produce a
 816 complete bifurcation diagram of the system, we have used here a
 817 small diversity in the α parameter values of different oscillators
 818 in the range of 10^{-3} . This does not qualitatively change the re-
 819 sults, but is a sufficient condition to obtain the complete bifur-
 820 cation structure of the system. For convenience, we have plotted
 821 here only one of the oscillators, although the full analysis has been
 822 performed.

823 *Figure 12.*

824 Top: time series of the protein B_i concentration in the regu-
 825 lar oscillating regime, exhibiting three cluster decompositions with
 826 7 : 6 : 5 distribution of cells between them. After a transient, a syn-
 827 chronous behavior inside each cluster emerges and the individual
 828 dynamics of the cells inside each cluster are indistinguishable. Bot-
 829 tom: cluster-plot representation of the case above. The parameters
 830 are those of Fig. 2, except $Q = 0.3$.

831 *Figure 13.*

832 Cluster plot of $N = 11$ non-identical repressilators in the self-
 833 oscillatory regime. A small diversity in parameter α_i makes the
 834 oscillators non-identical. α_i increases from bottom to top. The
 835 parameters are: $n = 2.6$, $\alpha_i = 210, 211 \dots 219, 220$, $\beta_a = 0.85$, $\beta_b = 0.1$,
 836 $\beta_c = 0.1$, $\kappa = 25$, $k_{s0} = 1.0$, $k_{s1} = 0.01$, $\eta = 2.0$, and $Q = 0.5$.

837 *Figure 14.*

838 Maximal Lyapunov exponent (top) and a numerical bifurcation
839 plot for the full amplitude oscillations (bottom) for increasing cou-
840 pling Q . The simulations have small genetic noise $\sigma_a^2 = 10^{-8}$ to avoid
841 tracking unstable orbits. $N = 18$, and other parameters are as in
842 **Fig. 2.**

843 *Figure 15.*

844 Time series (top panels) and the corresponding cluster plots
845 (bottom panel) in the self-oscillatory regime of $N = 18$ oscillators
846 with weak chaotic behavior and long lasting grouping. Parameters
847 are: $n = 2.6$, $\alpha = 216$, $\beta_a = 0.85$, $\beta_b = 0.1$, $\beta_c = 0.1$, $\kappa = 25$, $k_{s0} = 1.0$,
848 $k_{s1} = 0.01$, $\eta = 2.0$, $\sigma_a^2 = 10^{-10}$, and $Q = 0.6$.

849 *Figure 16.*

850 Time series (top panels) and the corresponding cluster plot (bot-
851 tom panel) in the self-oscillatory regime for $N = 18$ oscillators with
852 strong chaotic dynamics and short-lived time grouping, for $N = 18$
853 repressilators. The parameters are: $n = 2.6$, $\alpha = 216$, $\beta_a = 0.85$,
854 $\beta_b = 0.1$, $\beta_c = 0.1$, $\kappa = 25$, $k_{s0} = 1.0$, $k_{s1} = 0.01$, $\eta = 2.0$, $\sigma_a^2 = 10^{-10}$, and
855 $Q = 0.75$.

Table 1: Examples of the dependence of the IHLC oscillation period on the oscillators distribution between the high- and low-protein levels for $N = 18$ and $Q = 0.2$.

# HIGH	# LOW	PHASE	FIGURE	PERIOD
17	1	-	7 left	≈ 31.7
16	2	equal	7 middle	≈ 32.9
15	3	equal	7 right	≈ 34.0
14	4	equal	8 left	≈ 35.3
14	4	complex	8 middle	≈ 35.3
13	5	complex	8 right	≈ 36.5

Table 2: Examples of the clustering of the full amplitude oscillations.

<u>Q</u>	<u>cluster</u>	<u>phase</u>	<u>period</u>
$N = 2$			
0.1	1:1	equal	51.3
0.15	1:1	equal	50.0
0.2	1:1	equal	49.4
0.3	1:1	equal	47.7
0.4	1:1	equal	46.0
0.5	1:1	equal	44.5
0.6	1:1	complex	<i>many</i>
$N = 3$			
0.1	1:1:1	equal	51.3
0.15	1:1:1	equal	50.7
0.2	1:1:1	equal	50.0
0.3	1:1:1	equal	48.8
0.4	1:1:1	equal	47.2
0.5	1:1:1	equal	46.1
0.6	1:1:1	complex	<i>many</i>

$N = 4$			
0.1	1:1:1:1	equal	51.3
0.15	1:1:1:1	equal	50.7
0.2	1:1:1:1	equal	50.1
0.3	1:1:1:1	equal	48.8
0.4	1:1:1:1	equal	47.6
	2:2	unstable	-
0.5	1:1:1:1	equal	46.0
	2:1:1	asymmetric	45.0
0.6	2:2	complex	<i>many</i>

$N = 5$			
0.0	-	-	52.7
0.15	2:2:1	equal	50.7
0.2	2:2:1	equal	50.0
0.3	2:2:1	equal	48.6
0.4	2:2:1	equal	47.2
0.5	2:2:1	equal	45.5
	3:2	unstable	-
0.6	2:2:1	equal	44.1

$N = 6$			
0.2	2:2:2	equal	50.1
	3:3	unstable	-
0.3	2:2:2	equal	48.4
0.4	2:2:2	equal	47.3
$N = 18$			
0.2	6:6:6	equal	50.0
	7:6:5	equal	50.0
	8:5:5	equal	50.0
	7:7:4	unstable	-
	8:6:4	unstable	-
	9:9	unstable	-
$N = 100$			
0.4	34:34:32	equal	47.2
	35:33:32	equal	47.2
	35:34:31	equal	47.2
	36:33:31	equal	47.1