# Allee effect in eukaryotic cell populations

**Igor Segota, Ariana Strandburg-Peshkin, Xiao-Qiao S. Zhou, Archana Rachakonda, Benjamin Yavitt, Catherine J. Lussenhop, Sungsu Lee, Kevin Tharratt, Amrish Deshmukh, Elisabeth Sebesta, Myron Zhang, Sharon Lau, Anthony Hazel and Carl Franck**

Laboratory of Atomic and Solid State Physics, Cornell University, Ithaca, NY 14853 (USA)

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***Abstract***

The suspension culture of cells at provides an opportunity to study the multicellular life at low cell densities. In our earlier work with the model eukaryote *Dictyostelium discoideum*, we argued that the familiar but not understood transition from slow-to-fast growth at low cell densities (lag-log transition) was a collective, Allee effect during which cells communicate their density to one another by means of collisions. In this work, through improved protocols and assays, we put the phenomenon on a much firmer biological basis and show two new results. First, by means of variable shear rate experiments we prove that, in contrast to our previous conclusions, cell collisions are not responsible for the slow-to-fast growth transition. Second, we provide evidence that the transition is in fact caused by endocrine signaling – an unidentified molecule is secreted into the extracellular medium and used as a chemical signal. In order to interpret these results we developed a chemical signaling theory and calculated the expected cell density at the transition. This estimate agrees with observations, however within a broad range of theoretical uncertainty. Next, we estimate the experimentally observable variation in lag times at the transition, by considering the uncertainty in the inoculation density, possible variations due to asynchronous cell population and the fluctuations in receptor-ligand binding. None of these explanations are sufficient to explain the observed wide range of variation in lag times among identically prepared samples.

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# I. Introduction

Across a wide variety of organisms, from simple bacteria to complex animals, one observes the Allee effect [1] – an increase in fractional (or per capita) population growth rate with increasing population number; for general reviews see e.g. [2] or [3]. The mechanisms behind this effect have been shown to differ from one organism to the next. Proposed mechanisms include the presence of a slow growing subpopulation whose size is controlled by modulating the amount of toxin in the environment (in bacteria) [4], chemical signaling to induce growth (in yeast) [5], fluctuations in numbers of males and females (in fish and other bisexual organisms) [3] and various other mechanisms (see a summary of mechanisms in [6]).

In our earlier work [7] we provided the evidence that an Allee effect gives rise to the well-known slow-to-fast transition (lag-log effect) in *Dictyostelium discoideum* (Dicty) amoebae and sought to understand the mechanism behind it [8], [9]. Since Dicty are unicellular organisms, one hopes the mechanism might be easier to understand here than in complex organisms, and possibly comparable to other unicellular organisms. When grown in suspension (shaken culture), Dicty cells grow at a roughly exponential rate with a doubling time of ~20 hours, until a density of about $1\times10^4$ cells/ml. From that point onward they divide with a faster exponential rate corresponding to a doubling time of ~10 hours. The biology literature regards the lag phase (slow exponential growth) as a period in which isolated cells are somehow adapting to a new environment [10]. This explanation may be correct, but begs questions about the mechanism and does not provide any quantitative predictions; see discussion and citations in [7]. In published laboratory guidelines for Dicty [11], suspension cell culture protocols emphasize the role of density. They point out that the lag phase can be avoided by culturing cells at densities always above $2\times10^4$ cells/ml. So, either the adaptation to a new environment still occurs but at a significantly faster rate than at a lower density (and is too fast to be observable), or there is actually no adaptation at all and the lag-log transition in this case is purely a density-dependent (Allee) effect. This leads us to conclude that the adaptation hypothesis is unsupported by experimental evidence.

In [7] we offered a collective explanation for the slow-to-fast transition based on cells sensing each other's presence through collisions (juxtacrine signaling). The focus of the current paper is to establish, through a much more extensive data set of cell density vs time measurements in closed containers, two conclusions: 1) the means of intercellular communication behind the growth transition is not due to collisions between cells, and 2) the production and transport of soluble growth factors serves as a density signal (endocrine signaling). If a soluble growth factor is secreted into the surrounding media, the media gets "conditioned" and we use the experiments based on such conditioned media to argue for a chemical signaling mechanism. We also show that significant variations in growth characteristics for densities below $10^4$ cells/ml are in fact a characteristic of this system and not an artifact of low density cell culture or an experimental error. While a rough chemical signaling theory can account for the threshold density of the transition, the uncertainties in the inoculation density, variations due to asynchronous cell population (each cell in a different stage of the cell cycle) and the fluctuations in receptor-ligand binding kinetics seem to be insufficient to explain the observed variation around that threshold among different samples.

The organization of this paper is as follows. We first present the evidence for the wide variety of growth kinetics in traditional large volume cultures (25 ml), based on typically 5-10 simultaneous runs. Second, we report on an experiment with many more (~80) small volume (0.6 ml) vials to confirm the slow-to-fast transition and show the large variation in the distribution of lag times. Following up on our earlier work, we use variable rate stirring experiments to demonstrate that cell collisions are not responsible for the intercellular communications behind the transition. Also we reconsider the interpretation of our previous data [7] for conditioned media experiments, and conclude that: i) the cell densities used were close to the transition density of $10^4$ cells/ml, ii) there were only several (6) samples and iii) as we recently realized, it was lacking necessary control experiments used to more precisely estimate the fast growth rate (samples started in the fast growing phase above $10^4$ cells/ml). With that in mind, we decided to repeat our earlier attempts to search for soluble growth factors and in contrast with that work, we find strong evidence for them. Finally, a rough theory is developed for the density and fluctuations in the transition based on chemical signaling.

## II. Cell growth kinetics

In order to explore the slow-to-fast growth transition, we inoculated bottles of fresh culture medium HL5 (details given in Materials and Methods) with various low densities of cells derived from log phase cell cultures. The growth kinetics (cell density vs. time) were monitored and results are shown in Figure 1, for two common strains of axenic cells. These strains were grown in a culture medium, without bacteria - their natural food source.

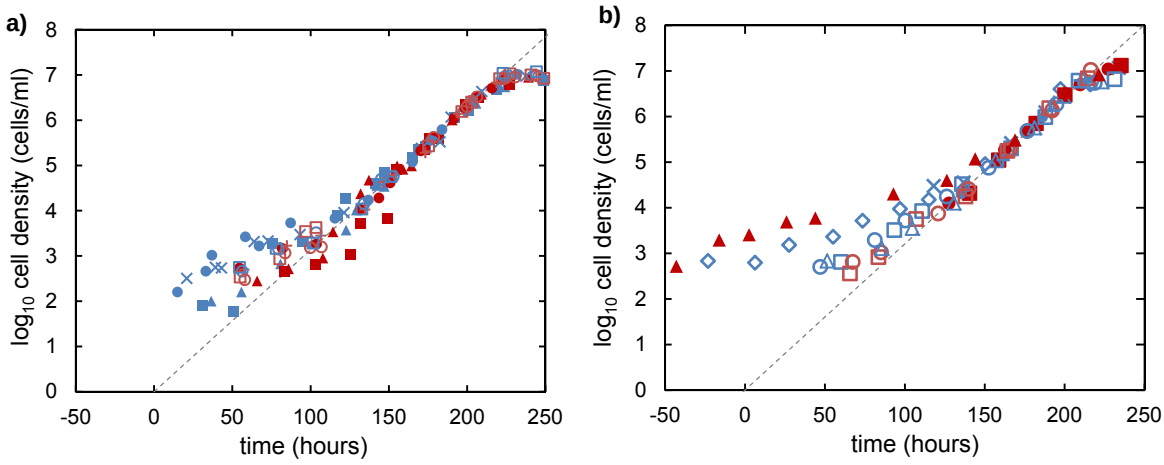



Figure 1. a) AX3 strain growth kinetics. Different symbols indicate different samples. Red points indicate samples where the source material used to start the culture was from the high end of the density range of exponential growth ($4\times10^6$ to $8\times10^6$ cells/ml), while blue symbols indicate the samples where the source material was taken from the low end of the exponential range ($5\times10^4$ to $3\times10^5$ cells/ml). The dashed line indicates the best alignment of exponential range data (from approximately $1\times10^4$ to $5\times10^6$ cell/ml) to a single exponential growth law by time translation of each run. The zero of time indicated is arbitrary. b) AX4 strain growth kinetics. Symbol color designations and alignment of runs to an exponential growth are as in part a). The particular source ranges for blue points were $2\times10^5$ to $6\times10^5$

cells/ml and for red points were $1.2\times10^6$ to $3\times10^6$ cells/ml.

In contrast to the smaller data set collected for the work in [7] where we presented three runs with starting densities below $10^4$ cells/ml (as opposed to the 18 shown here in Fig. 1), we see that there is considerable variation in the growth curves for densities below $10^5$ cells/ml. There is a single extraordinary run (solid red squares) in Fig. 1a, which shows neither lag-then-exponential nor completely exponential behavior, but rather grows even more quickly between $10^3$ and $10^4$ cells/ml than in the exponential regime of higher densities. Most importantly, in contrast to our earlier observations from which we concluded that a single universal density vs. time curve gave a good approximation of the observations for all comparable densities, we now reveal a continuum of behaviors from clearly lagging to apparently lagless growth at low densities. The theory we presented earlier can no longer completely account for our observations since it requires the assumption that there is a single universal growth density vs. time relationship. However, a vital density feature persists. We see that at around $10^4$ cells/ml, the growth curves all enter the exponential regime and remain at a consistently increased growth rate until the stationary phase. The doubling time shown in the exponential regime for both AX3 and AX4 (Figure 1a and 1b respectively) is about 9.6 hours. Here, we tested whether the source density had any effect on the slow-to-fast transition and found out that it does not. In order to confirm our findings and gain better statistics, we performed: i) another set of 11 experiments in large volumes (25 ml), with all samples started at the same density of 200 cells/ml, well below the transition density of $10^4$ cells/ml, (Figure 1a) and ii) a set of 77 small volume (0.6 ml) vials (see Materials & Methods for details) used for estimating the probability distribution of lag times (Figure 2b and 2c). Each set of samples was taken from the same source and was grown simultaneously. The lag time was determined by extrapolating the range from $10^4$ to $10^6$ cells/ml for each growth curve, and comparing to the starting time of cell growth (Figure 2b).

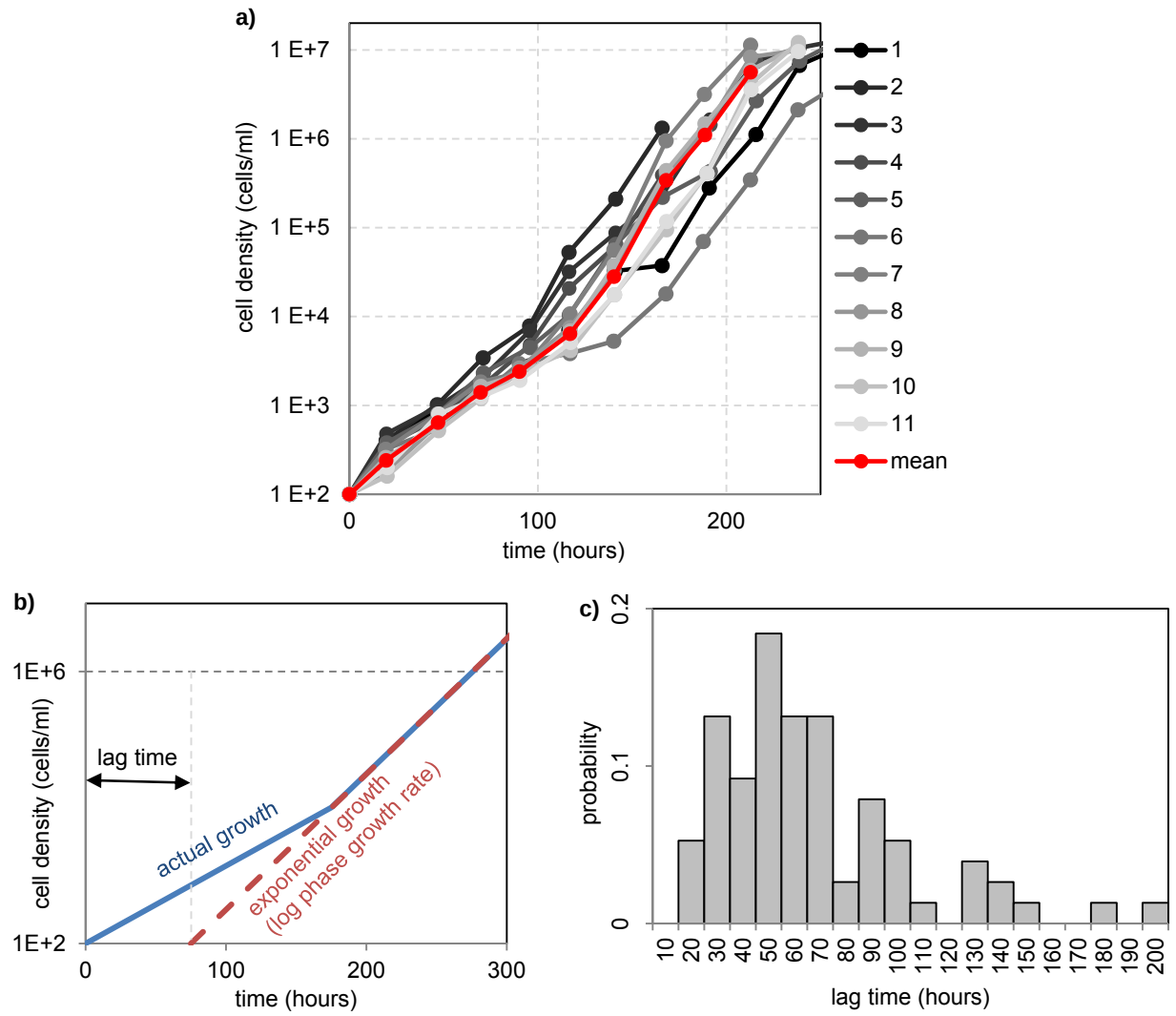



Figure 2. a) Growth kinetics of 11 samples grown in standard 25ml culture bottles, with the mean for each point shown in red. b) Procedure for determining and the definition of lag time. c) Probability distribution of lag times, based on the small volume vial experiment with 77 samples.

The slow to fast growth transition is clearly evident at about $10^4$ cells/ml when looking at the mean of all our samples (red curve in Fig. 2a). Using a nonlinear least-square fit for the mean of our data, we find doubling times for the lag phase: $T_{lag}$ = 19.5 ± 0.1 hours, and log phase $T_{log}$ = 9.49 ± 0.03 hours, with small relative errors, implying a good fit.

## III. Controls for effects of experimental artifacts

The wide range of variation seen in the growth kinetics curves of Fig. 1a and 1b caused us to examine more thoroughly the experimental conditions we employed, in order to check for any artifactual effect responsible for lagging. Following suggestions from [12], we employed a number of procedures to improve the sterility of our culture room (see Materials and Methods for details).

Our first concern was the possibility that the uncertainty in the initial inoculation density could lead to this variation. We considered the variation in lag times that could occur due to sampling uncertainty (shot noise) of low number of cells. For our small vials with 0.6 ml volume and the starting density of 200 cells/ml, one standard deviation of densities ranged from $n_{low} = \left(200/0.6 - \sqrt{200/0.6}\right)$ cells/ml $=182$ cells/ml to $n_{high} = \left(200/0.6 + \sqrt{200/0.6}\right)$ cells/ml $= 218$ cells/ml. Using the doubling time of 20 hours for the lag phase, we find the range of times to reach the transition density for each of these densities: $t_{low} = T_{1/2} log_2(10^4/n_{low}) = 116$ hours, and $t_{high} = T_{1/2} log_2(10^4/n_{high}) = 110$ hours. This gives the range of about 6 hours, however from Fig. 2c we observe a much larger range of variation. For our large volume flasks (25 ml), the spread would be even lower since the sampling uncertainty is much smaller. Therefore, the uncertainties in the inoculation densities cannot account for the observed range of variation in lag times.

Next, we considered the possibility that a low-level infection by bacteria might retard growth at low densities and that Dicty might combat this infection collectively, eventually winning out, and thereby producing an artifactual lagging effect. We disregarded results when a rare infection appeared in a sample and over took a culture. The frequency of these infections in our vial experiments was about 1 infection per 600 vials. What we were concerned with here, are contaminations that would escape detection in this manner. In tracking down a possible bacterial contamination, we noticed the occasional appearance of bacteria in our cultures. We discovered them occasionally in both our own cultures and in suspension cultures we received (unopened) from the Dicty Stock Center. The contaminant was identified as a strain of E. coli [13].

Through colony growth studies on agar plates we established that this strain was resistant to both antibiotics that we used, penicillin and streptomycin (PenStrep), but highly susceptible to tetracycline. In order to check for the effects due to bacterial contamination on the lagging phenomena, we performed the small volume vial lag time measurements with variable strength antibiotic doses. An antibiotic dose of 1 is defined as a dose of 25 μg/ml used previously [14], [15]. The dose of tetracycline is not standardized in the field as is PenStrep, as tetracycline affects both Dicty and bacterial growth, by inhibiting protein synthesis.

Rather than measuring growth curves with many points as a function of time, we inoculated many small volume vials (0.6 ml) with an initial density of 100 cells/ml, checked their turbidity daily and counted them when turbidity measurements indicated that they had densities in the vicinity of $1\times10^6$ cells/ml. Further, 3 samples for each dose were initially inoculated in the log phase at $10^4$ cell/ml. These provided the measurements of the growth rate since they were started above the transition threshold. By comparing the expected time for the initially low density specimens to reach $10^6$ cells/ml, we found the lagging time. Besides the advantage of being able to run many more specimens compared to conventional

large volume experiments, these vial measurements were especially immune to contamination since they were opened only once following inoculation. The results of these experiments are shown in Table 1.

Table 1. Log phase doubling times and average lag times for experiments performed with two different antibiotics (Penicillin/Streptomycin and Tetracycline) to check for weak effects of bacterial contamination. For each dosage we prepared 8 samples, as well as 2 control samples with no cells and 3 samples at $10^4$ cell/ml for log phase growth rate estimation.

| antibiotic | dosage | log phase doubling time (hours) | average lag time and standard deviation (hours) |
|---|---|---|---|
| PenStrep | 0 | 9.4 | 15 ± 12 |
| | 0.25 | 9.7 | 15 ± 8 |
| | 1 | 10.6 | 17 ± 8 |
| | 4 | 12.1 | 16 ± 3 |
| Tetracycline | 0 | 8.9 | 23 ± 12 |
| | 0.25 | 9.2 | 20 ± 11 |
| | 1 | 9.6 | 27 ± 19 |
| | 4 | 15.2 | -1 ± 6 |

We generally see no enhanced lagging with decreased dosage. The only anomaly we noticed is the lag time at the highest dosage of tetracycline, which appears to significantly reduce the Dicty doubling time. As mentioned, this is not unexpected since its mechanism of action affects Dicty as well, possibly eliminating the log phase altogether. Therefore, based on the fact that lag times do not change significantly with these variable amounts of antibiotics, we conclude that slow growth was not the result of bacterial contamination at low densities of Dicty.

## IV. Variable stir rate tests for cell collision mechanism

In our earlier work [7], we argued that cell-cell collisions were responsible for triggering the slow-to-fast transition and developed a theory predicting the growth curves based on two parameters: the critical number of cell collisions each cell undergoes before switching to fast growth ($N_C$) and the measurement time ($T_m$). If a slow growing cell acquires $N_C$ or more collisions during the measurement time $T_m$, the theory then predicts it will switch to fast growing cell. Here, we directly tested the validity of this theory by measuring the growth curves with varying cell-cell collision rate. In our small volume experiment with vials, we employ a multi-vial container rotating at a fixed rate. This design is a significant improvement for this purpose from the standard bottle shaker with inertial flow, because the flow in this setup is laminar (see Materials & Methods for details). The collision rate can be easily changed by changing the rotation speed (stir rate) of the container. The theoretical parameters $T_m$ and $N_C$ were adjusted to best fit the data obtained at a stir rate of 32 rpm. Using the same parameters, the theoretical growth curve was calculated and plotted, for stir rates of 16 rpm and 64 rpm (Figure 3).

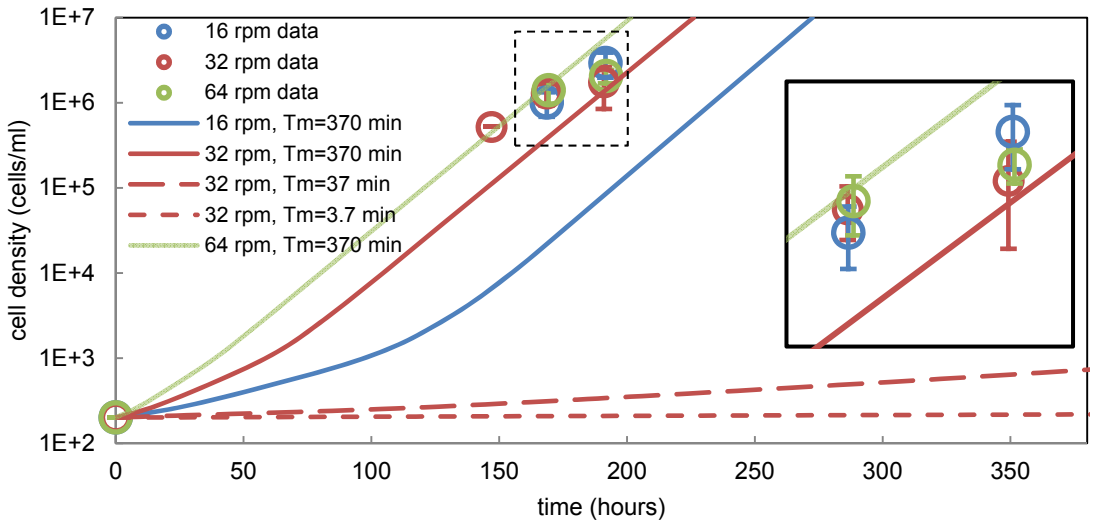



Figure 3. Fit of the experimental data to the cell-cell collision theory for three different stir rates: 16, 32 and 64 rpm. The parameters used for calibration were: measurement time $T_m$ = 370 minutes (~6 hours) and critical number of collisions to switch to fast growth $N_C$ = 1. If the measurement time is set lower, a fit cannot be made (see dashed lines for $T_m$ = 37 min and $T_m$ = 3.7 min). Once these parameters were set for stir rate of 32 rpm, stir rate was changed to 16 rpm and 64 rpm. Inset magnifies the area boxed in a dashed rectangle. All samples started from 200 cells/ml.

Changing another parameter $N_C$ (=1) to higher values would also extend the lag phase. However, in order to achieve a fit between the experiment and theory for larger $N_C$ , a measurement time $T_m$ required would be even higher than the current value of 370 minutes and would no longer be biologically meaningful.

We find three problems with the cell-cell collision theory. First, the only way the theory can be fit is to use a very long measurement time of around 370 minutes (6 hours). When compared to Dicty generation time of 12 hours, this result does not seem biologically plausible. Second, even if this is true,

the theory fails to match the obtained data for stir rate of 16 rpm. Third, the theory predicts significant discrepancies in lag times for the stir rates of 16, 32 and 64 rpm, which is not observed in experiments. Theoretical predictions and experimental results are summarized in Table 2.

| stir rate (rpm) | experimental lag time (hours) | theoretical lag time (hours) |
|---|---|---|
| 16 | 22 ± 7 | 80 ± 5 |
| 32 | 18 ± 9 | 25 ± 5 |
| 64 | 25 ± 14 | 0 ± 5 |

Table 2: Lagging results for variable stir rate experiments vs theoretical predictions. The experimental lag times are given as mean ± standard deviation of the sample. Theoretical lag times are given as mean ± error in the estimation by method shown in Fig. 2b.

Therefore, we conclude that the collision theory fails these three critical tests, and we must therefore consider a different mechanism for the slow-to-fast transition.

## V. Conditioned media experiments

Confronted with the failure of the collision theory for cell signaling, we return to an original hypothesis that cells are communicating by means of endocrine signaling – i.e. growth factors produced by cells are quickly dispersed in the well mixed suspension and stimulate other cells to grow if the concentration is sufficiently great. We performed a conditioned media test of this hypothesis. The endocrine signaling hypothesis predicts a positive conditioned medium effect. In other words, cells inoculated into media taken from cell cultures growing at high density (conditioned media) should show an increase in growth rate (and a decrease in lag time).

Previously [7], 8 experiments were performed in large volume (30 ml) samples. The initial cell density was between $2\times10^3$ and $7\times10^3$ cells/ml and the growth media was prepared by mixing equal (50%) amount of fresh media and conditioned media (obtained from the cultures at $10^6$ cells/ml). As mentioned in the introduction we reconsider the interpretation of our previous data [7] for conditioned media experiments, since i) the cell densities used were close to the transition density of $10^4$ cells/ml, ii) there were only 6 samples and iii) as we recently realized, it was lacking necessary control experiments used to more precisely estimate the fast growth rate (samples started in the fast growing phase above $10^4$ cells/ml). In our earlier work, we also performed a flow-through experiment in which we scanned an important range of Peclet numbers and found that the doubling time for the growth rate was unaltered. The difficulty arising here is that this flow-through experiment was missing a control experiment indicating that lagging is observed on a 2D substrate. Given that this growth is not in a suspension, it could possibly involve a different biology that is not affected by the external endocrine signal. In sum, our previous results do not eliminate the possibility of an endocrine signaling mechanism being responsible for the observed Allee effect. We therefore returned to investigating this possibility in the experiments described as follows.

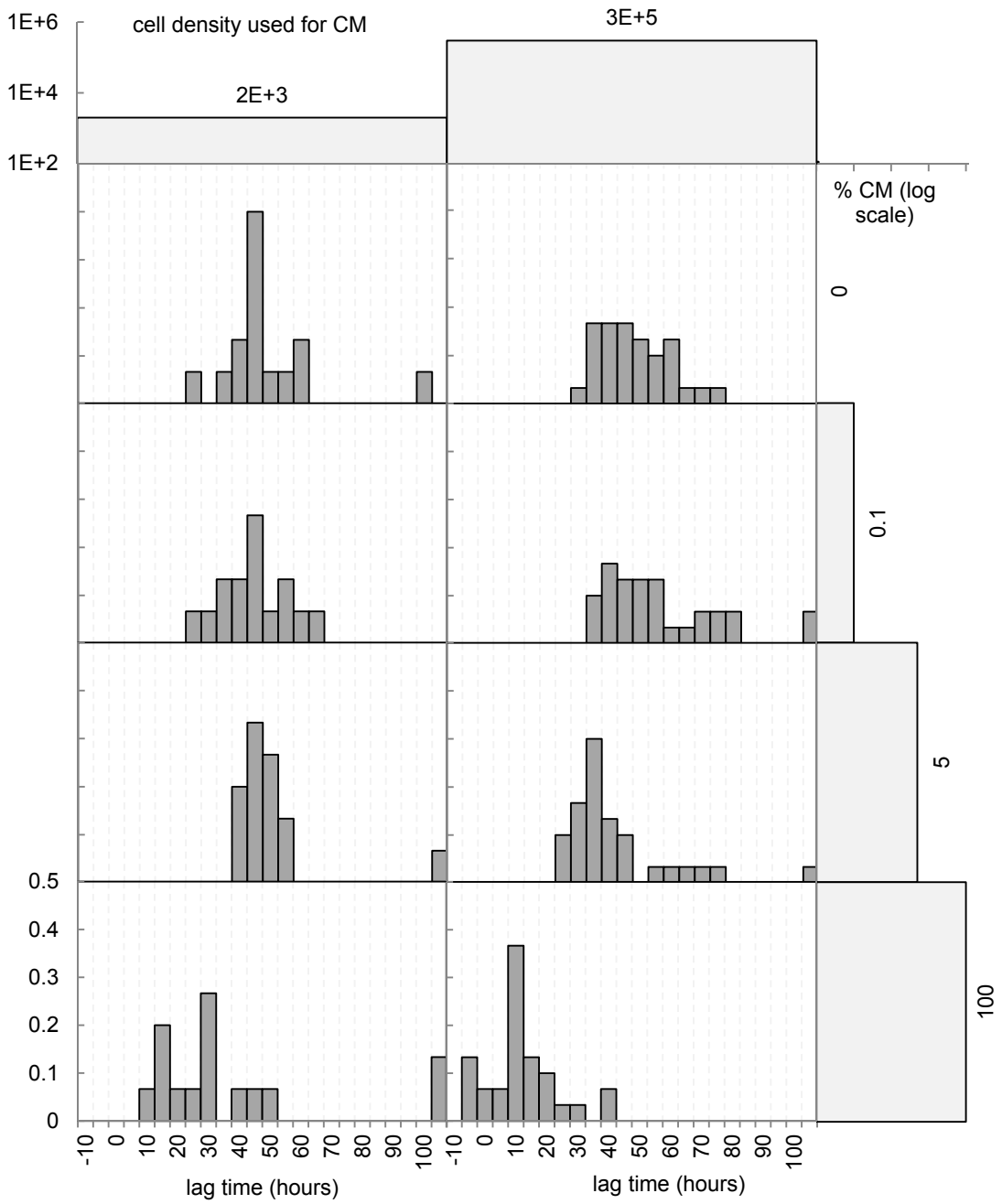



Figure 4. Probability distributions of lag times for all conditioned media (CM) experiments. Conditioned media was obtained from cells at two different densities: $2\times10^3$ cells/ml (left column) and $3\times10^5$ cells/ml (right column). For each case, a fraction of CM was mixed with fresh media in fractions of 0%, 0.1%, 5% and 100% (as indicated on the right). The resulting media was then used to grow cells and lag times were calculated according to procedure described in section II and Fig. 2b. Negative lag times correspond to samples growing faster than controls used to estimate the fast growth rate. The last bin on all histograms corresponds to lag times over 100 hours, i.e. very long laggers.

Rather than simply use 0% and 50% conditioned media, with 8 specimens each, we used 0%, 0.1%, 5% and 100% conditioned media with 26 samples each, and with conditioned media prepared from two initial

densities: $2\times10^3$ cells/ml and $3\times10^5$ cells/ml. The former, $2\times10^3$ cells/ml, was obtained by culturing cells started at $10^3$ cells/ml in fresh culture medium for 22 hours on a 150RPM shaker. The latter $3\times10^5$ cells/ml culture, was obtained by starting at $10^4$ cells/ml and growing them for 72 hours. In addition, all samples started from the initial density of 500 cells/ml. The results are summarized in Fig. 4. For each set of 13 samples at 500 cells/ml, we used two control samples initially inoculated with $5\times10^4$ cells/ml. These control samples were prepared for each concentration of conditioned media, and were used to deduce the log phase doubling time against which lagging was measured according to the same procedure as Fig. 2b.

Table 3. Lag time of the set of conditioned media experiments. The first four rows correspond to the experiments in the first column in Fig. 4 (conditioned media from $2\times10^3$ cells/ml density cells) and the second set of four rows corresponds to the second column in Fig. 4 (conditioned media from $3\times10^5$ cells/ml density cells).

| % CM | lag time (hours) | range of lag times min–max | number of samples |
|---|---|---|---|
| conditioned media from $2\times10^3$ cells/ml density cells | | | |
| 0.0 | 47 ± 17 | 21–99 | 15 |
| 0.1 | 43 ± 11 | 24–62 | 15 |
| 5.0 | 45 ± 5 | 38–54 | 14 |
| 100.0 | 37 ± 34 | 8–119 | 15 |
| conditioned media from $3\times10^5$ cells/ml density cells | | | |
| 0.0 | 46 ± 11 | 29–74 | 30 |
| 0.1 | 60 ± 41 | 32–244 | 30 |
| 5.0 | 45 ± 41 | 23–253 | 30 |
| 100.0 | 9 ± 12 | -9–38 | 30 |

The measured lag times are shown in Table 3. There is a strong reduction in average lag times between 0% and 100% conditioned media for the set of samples where we took conditioned media from cells at $3\times10^5$ cells/ml (above the transition), and somewhat weaker effect (if any) for the set of samples where we took conditioned media from cells at $2\times10^3$ cells/ml (below the transition). This supports the idea that one needs conditioned media from high density cells (higher than the transition density) in order to observe this effect. The negative lag times in Fig. 4 and Table 4, indicate cells growing with a faster rate than control samples, which were grown in parallel.

In summary, the lack of a stir rate effect on lag time distribution and the positive result from conditioned media experiments, lead us to conclude that endocrine signaling is responsible for the slow-to-fast transition observed in Dicty cultures.

## VI. Chemical signaling theory

Based on our experimental results indicating that chemical signaling is responsible for the Allee effect in Dicty cultures, we thereby consider a chemical signaling theory for the expected cell density at which the slow-to-fast transition occurs. Assuming that growth factor is secreted by cells, we assume its rate of production is proportional to the cell density $n$ and its decay rate is negligible in comparison:

$$\begin{aligned} \frac{d}{dt}c(t) &= \upsilon n(t) \\ \frac{d}{dt}n(t) &= \gamma(n)n(t) \end{aligned} \qquad (1)$$

where $c$ is the concentration of unbound growth factor, $\upsilon$ a growth factor production rate and $\gamma(n)$ a density-dependent fractional growth rate. Given that cells are growing at a reduced rate at early times ($\gamma_{lag}$) and later times at a higher rate given by $\gamma_{log}$, the growth rate is assumed to behave like a switch:

$$\gamma(n) = \begin{cases} \gamma_{lag}, & n < n_x \\ \gamma_{log}, & n \geq n_x \end{cases} \qquad (2)$$

where $n_x$ is the crossover density for the slow-to-fast transition. The initial conditions are $n(t=0) = n_0$ and $c(t=0) = 0$. The value for ν will be estimated based on two different examples from literature (see below). For cell densities $n < n_x$, $n(t)$ is given by:

$$n(t) = n_0 e^{\gamma_{lag} t} \quad (3)$$

Integrating (1), the cell density and growth factor concentration at the transition are given by:

$$c(t = t_x) = c_x = \frac{\upsilon n_0}{\gamma_{lag}}(e^{\gamma_{lag} t_x} - 1) \qquad (4a)$$

$$n(t = t_x) = n_0 e^{\gamma_{lag} t_x} \qquad (4b)$$

where $t_x$ is the time for the transition. Combining (4a) and (4b), we have:

$$c_x = \frac{\upsilon}{\gamma_{lag}}(n_x - n_0) \approx \frac{\upsilon}{\gamma_{lag}} n_x \text{ , for } n_x \gg n_0 \qquad (5)$$

Based on empirical results, we estimate $n_x = 10^4$ cells/ml, $n_0 = 10^2$ cells/ml and $\gamma_{lag} = ln2/T_{0.5}$ ($T_{0.5}$ is the doubling time in the lag phase). In addition we estimate the growth factor production rate per cell, $\upsilon$ to be in the range 400 – 9000 molecules/(cell×s).

Since the production rate $\upsilon$ is not measured for this growth factor, our estimate of it is based on an estimation of production rate of extracellular secretions from two related cellular systems: 1) the production of cAMP of starved Dicty and 2) the production of fibroblast growth factor by 3T3 cells. First,

in the Dicty starvation system authors in [16] monitored extracellular cAMP production from a high-density system which was pulsatile in nature. We averaged the production rate from their work, giving us the high value of $9\times10^3$ molecules/(cell×s). Second, although it was not intended to be quantitative as a measurement of growth factor secretion rate ν, authors in [17] observed the production of fibroblast growth factor due to heat shock. Assuming that a single standard size petri dish was used and estimating cellular density at confluence, we find production rate of 400 molecules/(cell×s).

Using the experimental data (Fig. 2a) we found $\gamma_{lag} \approx 0.5\gamma_{log}$, so the growth factor concentration at the transition, $c_x$ is between 8E-10 M (0.8 nM) and 2E-8 M (20 nM). Let us compare this value of $c_x$ to estimates for various receptor-ligand dissociation constants, $K_D$.

$K_D$ values for various growth factors ranges from 2E-8 (20nM) to 8E-11 M (80 pM) where typically $K_D$ = 5E-10 M = 0.5 nM [18]. So we conclude that $c_x/K_D = 0.03$ to 170 (most likely 1 to 28). Compared to a typical ratio for an Epidermal Growth Factor, $c_x/K_D = 0.01$ to 2 [19]. Based on this analysis, we conclude that within very broad limits, endocrine signaling can certainly be possible as a mechanism for slow-to-fast growth transition.

What could account for the observed variation in the transition time in Fig. 1a and 1c?

1. The fluctuations in inoculation density – as shown in section III, even for our smallest volumes, this does not account for the observed range of variation.
2. The variation in cell cycle (12 hours) could explain some variation in the transition time, but is not sufficient to explain our result that some cultures lagged for a period of several times that of the cell cycle time.
3. The variation in the growth curves due to chemical binding fluctuations of growth factor binding to its receptor.

We explore the last possibility below. Based on [18], the average and the variation in probability of occupancy of a receptor (θ) is given as follows:

$$\theta = \frac{c}{K_D + c} \quad (6)$$

$$\sigma_\theta^2 = \frac{cK_D}{R_T(K_D + c)^2} \quad (7)$$

where $R_T$ is the total number of receptors per cell. How do these fluctuations affect the value of c at the transition?

Differentiating (7) and using error-propagation, we obtain:

$$\sigma_{c_x} = \left|\frac{dc}{d\theta}\right|_{c=K_D} \times \sigma_\theta(c = K_D) = \frac{2K_D}{R_T^{1/2}} \quad (8)$$

This in turn provides the fluctuation in the expected transition cell density through the relation:

$$c_x = \frac{\upsilon}{\gamma_{lag}} n_x \quad (9)$$

and this gives us:

$$\frac{\sigma_{n_x}}{n_x} = \frac{2}{R_T^{1/2}} \quad (10)$$

Typical numbers for total receptor number per cell are [18] $R_T$ = 900 to $7\times10^5$ receptors/cell (typically $3\times10^4$ such as for epidermal growth factor). Therefore, the relative variation at the transition is:

$$\frac{\sigma_{n_x}}{n_x} = 0.0024 \text{ to } 0.066 \text{ (typical } 0.012).$$

Based on the experimental data, we estimate the variation in lagging is at least 10-15 hours (an underestimate). This implies an observed range of:

$$\frac{\sigma_{n_x}}{n_x} = \text{at least } 0.4 \text{ to } 0.6$$

This is considerably greater (40-60%) than the theoretical estimate given above (0.2-7%). Relaxing the requirement for $c_x = K_D$, we find the concentration of the growth factor at the transition would have to be:

$$\frac{c_x}{K_D} < 1.4\times10^{-4} \text{ or } > 80$$

Either option does not seems likely, however we do note that in our other work on folic acid chemotaxis, we observe that a response can occur even at $c/K_D \approx 100$ [20].

## Conclusions

This work studies a density-dependent growth transition at low densities in *Dictyostelium discoideum* amoeba. Unlike the well investigated starvation response aggregation of Dicty at much higher cell densities (~$10^6$ cells/ml), our attention was focused on the low density effect, reminiscent of the Allee effect displayed by many other single-cellular, animal and plant species.

With the goal of finding the mechanism of this process, we began by revisiting our earlier work [7], and using a more appropriate experimental vial system, we found strong evidence against the earlier proposed cell-cell collision (juxtacrine signaling) mechanism. In doing so, we were required to significantly improve our methods, paying special attention and investing extensive work to eliminate any possibility of the contaminations and ascertaining that the slow-to-fast transition is not a consequence of a low-level bacterial infection. The high variation in the slow-to-fast growth transition also required developing a batch method of doing many experiments in parallel, in order to establish a distribution of lag times.

The major conclusion of the work here is that the slow-to-fast transition during cell growth occurs by a chemical signaling mechanism. This follows immediately from conditioned media experiments, which show a significant positive result, indicating that the growth media is changed during cell growth (e.g. cells secrete growth factor into their environment). The chemical signaling theory developed here predicts the correct range of growth factor concentrations within very broad limits; however it also predicts rather extreme concentrations of growth factors if we assume that chemical binding fluctuations are responsible for the observed variation in transition times. Even though we cannot completely dismiss the possibility of having growth factor concentration in this extreme range, we conclude that further work is necessary to explain the observed variation in growth transition.

Considerable progress has been made recently on a parallel front, in discovering collective effects in bacterial colonies due to endocrine signaling. Our investigation here of the slow-to-fast transition

represents a part of the effort to understand the nature and consequences of such “quorum sensing”. [21] In another synthetic realm, modern telecommunications is looking more and more biological with the appearance of ad hoc networks. We can anticipate that the power of contemporary telecommunications theory will be brought to bear on living systems. [22] By the same token, perhaps biology will continue to return the favor with new designs for human telecommunications.

Future work branches into four directions. First, there is a possibility of memory effects [23] in slow-to-fast transition and each state persisting through generations (e.g. phenotypic switch). The hypothesis here is that the epigenetic mechanism could be possibly causing the wide variation in transition times and leaving cells in one (slow growth) or the other (fast growth) state. A series of experiments could be run in an attempt to determine if it is possible to select for fast (or slow) growing cells by repeatedly selecting for the fastest (or slowest) growing samples and culturing them further through several generations. Second, there is still an open question whether a completely lagging (consistently slow growing) or lagless (consistently fast growing) strain can be isolated and there the future work could consist of growing monoclonal Dicty colonies (growing an entire colony from a single cell) in batch. This effort could also provide a way to estimate the effect of genetic diversity on the observed variation in lag times. Third, if the slow-to-fast transition could be observed on a substrate in 2D this could lead to more direct observations. For example, if the slow growing cells got stuck in a particular phase of a cell cycle, a Dicty strain with a GFP marker for a particular phase of the cell cycle [24] could be used to observe the doubling time for each individual cell. In addition, one could easily observe and quantify any possible correlation between the doubling time and local cell surface density. Finally, given that the complete Dicty genome has been sequenced and is available, there is an opportunity to look for unidentified growth factors within the genomic sequence and an invitation to attempt to purify the secreted growth factor.

## VII. Materials and methods

**Cell growth and growth kinetics**

Inoculation of shaker bottles: As described in [7], cell culture followed a standard protocol for this organism in [11] and [25], modified by the addition of antibiotics and extended subculturing. Closed flasks containing 25 ml of HL5 culture media [26] treated with 2500 units of penicillin and 2.5 mg of streptomycin (250 μl per 25 ml bottle) were shaken constantly at 150 rpm on a rotary shaker after inoculation from exponentially growing cultures. We continually subcultured for a year noticing no systematic variation over that period. In the course of propagating cells, cultures were sometimes allowed to enter the stationary phase or were unshaken for periods of time. In such cases, subsequent cultures were not used for the growth studies reported here until they were subcultured so as to pass through the exponential phase but not reach the stationary phase.

We point out in [7] about the possibility of obtaining a lagless strain. In scanning for examples of this (surprisingly) lagless behavior, we examined lines derived from frozen samples of the original strain that was used in the earlier work as well as material provided by the stock center for *Dictyostelium* research. In no cases were we able to establish consistent lagless behavior through repeated culture passages. Similarly, lagging cultures could on occasion be found to yield lagless behavior upon propagation.

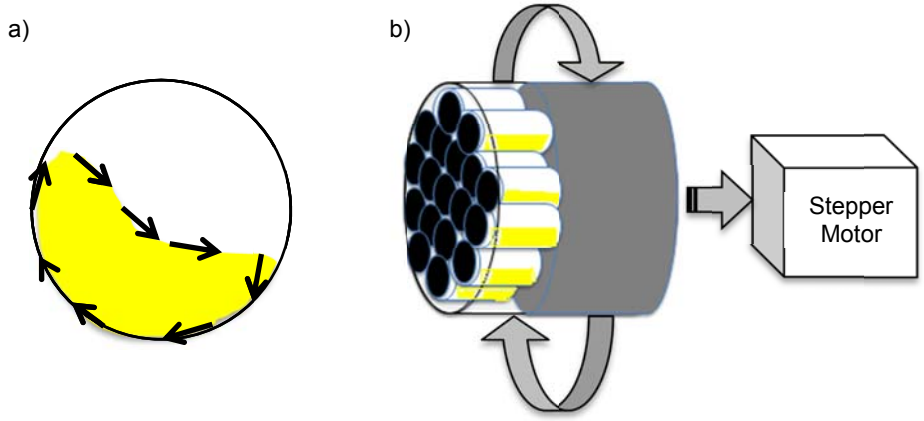
Measurement of cell density: Cell densities were taken using a counting chamber [27] with a volume 500 times greater than that provided in our original work. With this counting chamber, we eliminated the concentration step necessary in our earlier work and gained greater sensitivity in measuring cell density. We found by comparisons with cell-free containers of culture media that a false-positive background density corresponding to typically 60 to 180 cell/ml could be expected, presumably through misidentification of particles as cells within the culture media. The statistical counting uncertainties were typically 40% at 100 cell/ml, and 22% for densities 1,000 cells/ml and above. The total error could be estimated by looking at the spread in the first measured point in Fig. 2a. The cells were diluted to a concentration of 200 cells/ml, so the initial density is not measured but experimentally set.

Improvements in cell culturing: As a precaution in combating possible bacterial contamination, we only used penicillin & streptomycin that had been filtered before it was aliquoted from the supplier's container [17]. While for the observations plotted in Figures 1 and 2, we kept the room lights off in our culture room when it was not in use, we kept them on at all times in future experiments in order to suppress any possible effects of lighting on cell growth (such as entrainment of circadian rhythms) [28]. We improved the sterility of our clean culture table by running ultraviolet sterilizing lights when not in use. We repeatedly checked for the possible effects of extended subculturing by using fresh cells from the Dicty Stock Center every few months and newly thawed out cells from our frozen stock. As a final improvement, we increased the number of simultaneous observations by running several shaker bottle growth experiments in parallel with several small volume vial lag tests.

**Variable stir rate experiments**

While experiments conducted using traditional shakers, such as the one described above, allow for precise measurement of individual growth curves, culturing up many shakers and measuring them daily requires a considerable amount of experimenter time, and therefore sample size is limited. In an

alternative setup, 77 small vials, each containing 0.6 ml of cell suspension, were maintained in a rotating drum. Each day, we measured the turbidity of each vial to determine when the cell density reaches approximately $10^6$ cells/ml. When this turbidity was reached, we measured the cell density precisely using counting chambers.




The rotating drum was mounted on a stepping motor. Care was taken to operate the stepping motor at rotational speeds that did not produce vibrations. Running the stepper motor at too low rate (~ few rpm) would produce stepping motion which causes the flow within vials to be inertial and not smooth laminar. Running the motor too fast on the other hand, can introduce various mechanical resonances and again produce vibrations leading to different kind of (inertial) mixing.

Each day, turbidity of each vial in our drum mixer was checked using a system consisting of a red laser diode and phototransistor monitor in line with the laser beam (forward scattering). The system was calibrated with samples of cell densities, which were checked using a counting chamber. Note that in case of a rare bacterial infection during any time the vial would turn very turbid and we would be able to detect it within a day, discarding the vial.

**Conditioned media experiments**

Unlike in the original experiment where we used syringe filters to remove cells, in the present work we used centrifugation and verified that cells had been removed down to a level of 40-80 cell/ml by counting. Due to this uncertainty we started with initial densities of 500 cells/ml.

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