nature portfolio

# Peer Review File

# Metabolic coordination and phase transitions in spatially distributed multi-cellular systems

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**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done significant work including simulations, theory, and data analysis. However it is not very clear what has been accomplished by these efforts. I recommend a serious revision that would make it very clear what is novel in this work, which results one should try to verify experimentally, and what aspects are an artifact of the model.

1. I don't think the authors state clearly what the question they are trying to answer or hypothesis to test. Perhaps the closest they come to it is saying that overflow metabolism in tissue is not fully understood. I think the aim of the study should be stated very specifically. For example, a generic statement about emergence like in "This raises a rather basic question..." does not really help me understand what the paper is about.

2. The abstract states that the following are the main results.

Specifically, a phase transition from a balanced network of exchanges to an unbalanced overflow regime occurs as the mean cellular glucose and oxygen uptakes vary while single-cell metabolic phenotypes are highly heterogeneous around this transition. We then show that time-resolved data from human tumor-stroma cell co-cultures consistently map to this crossover region, supporting the idea that environmental deterioration reflects a failure of coordination among recurrently interacting cells.

I thin this means that. (i) the production of lactate changes from zero to a positive value as the uptake of glucose increases relative to the oxygen uptake, (ii) metabolic fluxes are somewhat different among the cells, (iii) tumors could have secreted a lot more lactate if they did not care about their growth rate.

Given the standard set of metabolic reaction only the (ii) is a non-trivial statement. Even then, the authors "explain" this by simply allowing metabolic fluxes to fluctuate. All-in-all, it is not clear to me what are the novel, experimentally testable, results in the manuscript.

The abstract ends with

In summary, our findings suggest that, rather than deriving from multiple independent cell-autonomous processes, environmental control is an emergent feature of multi-cellular systems.

There are similar grand-sounding statements throughout the manuscript often about "the breakdown of coordination". As far as I understand the work, all of the results are explained by the meanfield model, which assumes that each cell is independent except they all have access to the same pool of lactate in their environment. That is each cell has the same "strategy" and sees the same environment and these two factors are the only sources of collective behavior. I feel that such scenarios should be described in much less grandiose terms. For example, stochastic secretion of lactate is limited by negative feedback (cells consuming excess lactate) until majority of cells in the population switches to lactate production.

3. I also have some concerns about the simulations underlying the reported results.

I don't think the spatial distribution of cells adds much to the study because it gives the same results as the meanfield model

and depends on numerous additional and often unrealistic assumptions. The latter include diffusion of lactate, but not glucose, 3D diffusion of lactate, but 2D arrangement of cells, no temporal dynamics, etc.

One of the key assumptions is that all feasible points in F_N are equally accessible, i.e. have the same weight up to the Boltzmann factor. It is not clear how one would obtain such joint distribution from each cell following a Boltzmann distribution (perhaps dependent on the lactate concentration in the medium). My questions is whether this assumption adds an "invisible hand" that influences the dynamics in ways beyond what could be achieved by independent cells in common environment.

Another assumption is the choice of h(u). I think some previous works using this formalism assumed that h(u) is the growth rate rather than the incoming fluxes. Would this significantly change the results?

Finally, the parameterization in terms of beta has two effects. The first effect is that it switches the corresponding flux from minimal to maximal. The switching point is around beta=0. At the same time, beta controls the fluctuations, which are maximal around beta=0 as well. Thus when the authors report large fluctuations near the transition between coordinated and overflowing states, they potentially conflate two phenomena: the increase of fluctuations near a phase transition due to collective effects and the increase of microscopic fluctuations driven by the choice of the parameterization in terms of beta. In the physics language, this would correspond to choosing a temperature-dependent coupling in the Ising model such that J has a minimum at the critical temperature.

4. In Fig. 2, it would be interesting to see how the average u_L behaves as a function of beta and u_G for a single cell bathed in unlimited lactate. In particular, I wonder how the location and steepness of the collective transition differ from that in a single cell (presumably the single cell would also have a region of u_L=0 and u_L>0).

5. I wonder if the deviations between the meanfield and the spatial model are due to the fact of finite N rather than the effects of space.

6. I would double check the caption of Fig. 1B; I think some points are not correctly labeled.

7. The application of theory to cancer data has several problems. The first one is that the model assumed a steady state, but the cancer data has a very strong time-dependence. I don't think 1 hour time scare is enough for the equilibration of nutrients and metabolic fluxes. Moreover, the environmental concentration of lactate goes down, so there is a net consumption of lactate, which was forbidden in the theory.

Secondly, I don't understand why the accumulation of data on the boundary between the regions is surprising. In Fig. 4D, the other side of the boundary is inaccessible (net consumption of lactate). So a simple explanation would be that the cells just feel/grow best without excreting too much lactate. The accumulation near boundary in Fig. 4C could be an artifact of a very noisy experimental data (this noise could be measurement noise not the real heterogeneity) and the fact that the theory has the greatest noise near the transition. So in order to match the noise in the data, the parameters need to be near the transition.

Thirdly, I did not understand the reasoning behind Fig. 5. It seems that the authors say that low oxygen uptake results in high oxygen concentration, which is totally logical from the reactions. However, this means that the cell cannot have both low oxygen uptake and low oxygen concentration, which is the true state of hypoxia. I think many of the claims are based on the fact that the media is not hypoxic when the cells take up little oxygen, but such cells grow "hypoxically"!

8. Finally, any claim about collective effects should be made only after comparing the behavior of a single cell and a collection of cells. The single cell model should allow for a reservoir of lactate. That is at a given time step the cell can either uptake lactate from the reservoir (if it has some) or excrete some lactate that is stored in the reservoir. One can then plot the average lactate concentration in the reservoir as a function of N and based on that talk about collective behavior. It seems that at least some of the observations are due to the fact that there is a reservoir in the environment not some intricate coordination among the cells.

Reviewer #2

(Remarks to the Author)
In this paper, the authors study the phenomenon of overflow metabolism, a process that occurs in microbial communities as well as tumor microenvironments. A statistical and analytical framework is used to show that a phase transition occurs between a balanced state with low concentrations of potentially toxic overflow products, and an unbalanced overflow state marked by high heterogeneity of flux across cells. They then show that in an experimental co-culture of cancer cells and fibroblasts, the dynamics map to the critical line separating these phases. They argue that these results show that the state of the environment is an emergent effect of inter-cellular interactions.

The last point, about the emergent effects, is the most unclear of the paper's main claims. Overall, however, the paper is well-written and clear, with convincing explanations of the methods and results. The topic will be broadly interesting to researchers studying various systems. I have some suggestions for clarifying a few points, and for describing the results to those studying other systems.

First, a better description of the assumed cellular dynamics and behaviors would help. These results will interest microbial ecologists, who study overflow metabolism in the context of cellular growth and division, often in glucose-limited

environments. Does glucose import in this system lead to growth and division? Do the opposing strategies to optimize rate or yield of ATP (Fig. 2) correspond to r/K strategies (in yeast, these descriptions correspond exactly)? If the cells are not dividing, what do these strategies describe?

Furthermore, is glucose limiting in this system, and is it depleted over the time course of the experiments described? (Why isn't a glucose concentration time course shown, similar to those shown for lactate and oxygen?) The description (page 9, top of first column) of adaptation to the medium, where average glucose and oxygen import decrease over time, brings to mind a microbial culture reaching stationary phase after glucose has been depleted. Yet the authors describe this process as movement "toward the state of optimal ATP yield in the balanced regime." Are there any commonalities between these processes?

More generally, can this model be applied to different systems to understand overflow metabolism? While oxygen remains high in this system, what would the model explain about hypoxic tumor microenvironments or anaerobic fermentation? The phase diagram (Fig. 3B) suggests that transition to the overflow state is triggered more easily by glucose import in low-oxygen environments. Is this true, and if so, what is the relevance to high-lactate tumors and high-ethanol fermentation in which yeast overflow products lead their own populations to collapse?

Below are some more specific points:

- The model is described as two-dimensional. A justification for this assumption is missing, or a discussion of how results might change in three dimensions.
- "Our analytical expression for <u_L>" is mentioned on page 6, top of second column, but no expression is referenced.
- Why is lag phase, or the population's adaptation to the medium, important to study? And why does the time course begin at 1 hour, and not earlier?
- The description of the process of inferring lactate fluxes for Fig. 4 (page 7, first column) starts with previously obtained lactate fluxes, which are then used to infer beta_G and beta_O. This logic sounds circular.
- Saturated mitochondrial capacity should be defined in terms of the model (page 9, first column). In the current version of the text, it is unclear how the fraction of saturated mitochondrial, shown in Fig. 5, is obtained. I also couldn't find this in the supplement.
- In the Discussion, what results do statements about heterogeneity refer to? Should the reader refer to plots of variance in Fig. 3? This feels like an unexplained result.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am happy with the revision.

Reviewer #3

(Remarks to the Author)
I thank the authors for their thorough response. I enjoyed reading about all of the additions to the paper, and I have no objections. My only suggestion would be to describe the system of study more clearly, perhaps at the end of the Introduction. Since both Reviewer 1 and I both asked about the cellular growth rate, others will likely wonder about the dynamics. It would be helpful to include the description given in the reviewer response about how the cells are not growing or depleting glucose very much, and the reasons for studying the lag phase in the tumor microenvironment.

Dear Editor,

We sincerely appreciate your correspondence regarding our manuscript and extend our gratitude to the Reviewers for their insightful comments and suggestions. We have carefully revised the manuscript in response to their critiques, with substantial improvements, particularly in the statistical inference aspects of our work. Specifically:

- To reinforce our claims about the significance of intercellular interactions, we analyzed nearest-neighbor flux correlations in experimental data. We identified trends that cannot be replicated within isolated, single-cell models but can be captured by our two-dimensional spatial model of interacting cells. These additional data have now been incorporated into the inference pipeline.

- We refined our inference protocol by implementing a more rigorous treatment of the likelihood function and by comprehensively sampling the posterior distribution. This allowed us to derive error bars for the inferred model parameters. Additionally, we partitioned the data into training and test sets, validating the model's predictive capabilities and optimizing the hyperparameter regularization to enhance performance.

- We extended the model to account for the background lactate term, which we had previously assumed to be negligible. We now provide a thorough discussion of its theoretical implications—showing that it shifts the phase transition line, potentially eliminating it at high background levels—and its impact on our inference results. Our findings indicate that the system is near criticality only in the early stages of adaptation, after which it remains in a single phase (the 'Warburg phase') as the critical line shifts. Despite these refinements, the overall inferred experimental trajectory still follows a path of metabolic rate reduction (yield increase).

- Figures 4 and 5 in the main text have been redrawn, and the discussion on inference has been revised accordingly. Additionally, we have added five new sections to the Supporting Information to comprehensively address all concerns raised by the reviewers.

Changes made in response to the points raised are reported in red in the article text. Our replies to their comments follow in blue. Once again, we appreciate your time and consideration. We are confident that with these improvements, the manuscript now meets the required standards for publication.

Kind regards,

The Authors

# Reply to Reviewer 1

*The authors have done significant work including simulations, theory, and data analysis. However it is not very clear what has been accomplished by these efforts. I recommend a serious revision that would make it very clear what is novel in this work, which results one should try to verify experimentally, and what aspects are an artifact of the model.*

We thank the Reviewer for their detailed assessment, based upon which we have thoroughly revised the manuscript as described in detail below. The major point on which we focused in our revision was the inclusion of further empirical data in the inference pipeline, most notably (i) the background lactate level (as suggested by the Reviewer) and (ii) the nearest-neighbour cell-to-cell correlations of lactate fluxes. Both have a very significant impact on the results.

The former effectively works as an exogenous forcing field that acts on the population as a whole and whose intensity increases over time. We had neglected this term in our original submission upon incorrectly assuming that it is negligible over the entire duration of the dynamics. However, this only holds for slightly more than half of the 6-hour long experiment. As the Reviewer likely suspected (and as we now show in Supporting Information, Sec. S10), background lactate concentrations become increasingly relevant at later experimental time points. Here, purely dynamical effects dominate and the (static) mean-field model we originally used as a theoretical guideline to interpret inference results departs from inference results. We are therefore especially grateful to the Reviewer for suggesting we consider it. We devoted a new section in the Supporting Information, Sec. S7, to explaining how our framework changes in presence of background lactate. As a consequence, Figure 4 of the main text and the discussion around it have been substantially revised.

As for correlations, their inclusion in the inference pipeline turned out to be essential in order to represent the enlarged set of empirical data we are currently using in our inference protocol (now including the background lactate term). Most importantly, we show that the observed negative correlations in data cannot be reproduced with non-interacting cells, thereby pointing to the key role of inter-cellular interactions which lie at the core of our work. Two new sections in the Supporting Information, namely Secs S9 and S11, now focus on the role of nearest-neighbour correlations and on how they are accounted for in the inference protocol. Results and discussion in the main text have been extensively revised accordingly.

In view of the above, results presented in the main text under "Inverse modeling experimental data" have been substantially revised and improved.

In summary, the manuscript benefited greatly from the suggestions and criticisms of the Reviewer, allowing us to separate more clearly the relative contributions of cellular interactions, metabolic constraints, and changing environmental conditions in explaining the data. In addition, we have clarified the limitations of the equilibrium-based model we used to benchmark our results in the original submission. Ultimately, we believe that this revision highlights much more clearly the key novelty of our work, namely the quantitative appraisal of the role of cell-to-cell couplings in the adaptation dynamics of populations of cells.

Detailed point-by-point replies are given below.

1.1 *I don't think the authors state clearly what the question they are trying to answer or hypothesis to test. Perhaps the closest they come to it is saying that overflow metabolism in tissue is not fully understood. I think the aim of the study should be stated very specifically. For example, a generic statement about emergence like in "This raises a rather basic question..." does not really help me understand what the paper is about.*

We thank the Reviewer for raising this point. In short, the specific problem we address is theoretical in nature and concerns the role of intercellular interactions in shaping the

environment and adaptation dynamics of (low-density) populations of cells. In particular, we want to separate cell-autonomous effects, due e.g. to cellular metabolic constraints or idiosyncratic objective functions (where interactions do not matter and cells evolve independently) from effects that require a degree of coordination between cells (driven by interactions, either local, e.g. via nearest neighbours, or non-local). Such a question is central, we believe, both to understanding the adaptation dynamics of cell populations and improve models of cellular communities. If cell-autonomous effects dominate, populations can effectively be represented by a single 'average cell' (pursuing, most likely, the optimization of an objective function); if interactions dominate, this is not possible. Single-cell states that are not allowed in cell-autonomous models become feasible within populations. If interactions matter, models should therefore account for this additional degree of complexity. In the present revision, we try to make this point much more clearly than we did in our original submission, not only in terms of introductory statements but also in quantitative terms (through the extended inference pipeline that accounts for correlations). We have now tried to stress these aspects both in the Introduction and in the Discussion. We hope this clarifies the theoretical (main) goal of our study. Regarding the concrete system that we use as a testing ground, we chose the phenomenon of acid (lactate) accumulation in populations of mammalian cells sharing a glucose- and oxygen-rich medium (known as the Warburg effect when cancer cells are involved) because high-resolution empirical data that are ideal for our theoretical goal have been recently made available [Onesto et al, Probing Single-Cell Fermentation Fluxes and Exchange Networks via pH-Sensing Hybrid Nanofibers, ACS Nano (2023)]. The cellular sub-system playing the key role here is metabolism, and the relevant interactions correspond to exchanges of acids derived from the fermentation of glucose. Acid overflow at the level of single cells can be explained in terms of the sudden change in the solution to a constrained optimization problem, occurring when certain intra-cellular metabolic constraints are saturated [De Groot et al, The common message of constraint-based optimization approaches: overflow metabolism is caused by two growth-limiting constraints. Cell Molec Life Sciences (2020)]. In a population of non-interacting cells, lactate accumulation would follow from a multitude of independent events of this type. The working hypothesis of the paper is different: in a nutshell, in absence of external lactate sources, if exchanges of lactate are balanced among cells, i.e. if the metabolic states of cells are somehow coordinated, no lactate spillover occurs. By contrast, lactate accumulation is due to be observed whenever imbalances in the exchange network are present. As a consequence, in order to fully understand the origin of lactate overflow in mammalian cell cultures, inter-cellular interactions are essential and lactate excretion by single cells does not necessarily imply acidification of the medium. The evidence we present supports this scenario.

We finally stress that lactate exchanges have long ago been proposed as central for the onset of lactate overflow. This idea is perhaps most notably summarized by the lactate shuttling theory put forward by GA Brooks [The Science and Translation of Lactate Shuttle Theory, Cell Metabolism (2018)], which has recently been revisited and updated using new empirical insights [Lactate: the ugly duckling of energy metabolism, Nature Metab (2020)].

1.2 *The abstract states that the following are the main results:*

*Specifically, a phase transition from a balanced network of exchanges to an unbalanced overflow regime occurs as the mean cellular glucose and oxygen uptakes vary while single-cell metabolic phenotypes are highly heterogeneous around this transition. We then show that time-resolved data from human tumor-stroma cell co-cultures consistently map to this crossover region, supporting the idea that environmental deterioration reflects a failure of coordination among recurrently interacting cells.*

*I think this means that: (i) the production of lactate changes from zero to a positive value as the uptake of glucose increases relative to the oxygen uptake, (ii) metabolic fluxes are somewhat different among the cells, (iii) tumors could have secreted a lot more lactate if they did not care about their growth rate.*

*Given the standard set of metabolic reaction only the (ii) is a non-trivial statement. Even then, the authors "explain" this by simply allowing metabolic fluxes to fluctuate. All-in-all, it is not clear to me what are the novel, experimentally testable, results in the manuscript.*

In the light of the new inference results we include in this revision, the abstract has been adjusted to better reflect the most recent results. Specifically, given the fully dynamical nature of the data now employed in the inference pipeline, the static mean-field model we introduced in the original submission now only provides a qualitative benchmark for interpreting results. (This is explained in detail below.)

Regarding point (i) ("*the production of lactate changes from zero to a positive value as the uptake of glucose increases relative to the oxygen uptake*"), as far as we understand, in models of cellular metabolism the behaviour described by the Reviewer only occurs when one assumes that cells maximize their growth rate subject to some global constraint on fluxes (e.g. a finite respiratory capacity) [De Groot et al, The common message of constraint-based optimization approaches: overflow metabolism is caused by two growth-limiting constraints. Cell Molec Life Sciences (2020)]. In the present model, cells do not optimize anything. This is an essential point in our view. We also remark that 'production of lactate' makes sense for a single cell. Our results refer to the population-averaged lactate fluxes and presuppose the existence of cells that excrete lactate as well as cells that consume lactate. Again, this is a trivial but very important difference, especially in view of point (ii) ("*metabolic fluxes are somewhat different among the cells*"). Indeed, fluxes are different among cells, but in a correlated way. That is, inter-cellular differences cannot be interpreted as "noise", "random fluctuations" etc. Concerning point (iii) ("*tumors could have secreted a lot more lactate if they did not care about their growth rate*"), it is not totally clear to us that this is the case. In a cell-autonomous scenario it would actually seem to us to be the opposite. But the issue of what cells do versus what they could do is, we believe, a rather complicated one where inhomogeneities in cellular microenvironments are crucial. With better data it may be possible to infer 'simple behavioural rules' for single cells, that could be used for instance to construct dynamical agent-based models to describe such systems.

We thank the Reviewer for pointing out the weakness in our original presentation concerning predictive capacity and testable predictions. As for the latter, in short, the finite-dimensional model we present (which we can unfortunately only study via simulations) is ideally suited to analyze a broad spectrum of observables that are experimentally accessible. For instance, we show that it can quantitatively reproduce the statistics of single cell lactate flux data including intercellular metabolic correlations. This is, we believe, non trivial, because nearest-neighbor correlations are impossible to reproduce within a non-interacting model (see Figure 4 and new sections S9 and S11 in the Supporting Information, as well as the detailed discussion in the main text after equation (18)), and they are much weaker (and independent on position) in the mean-field approximation. Furthermore, the revised main text Section entitled "Mitochondrial saturation versus local hypoxia: dynamics of oxygen usage" contains a host of results about oxygen usage by cells that are in principle testable in experiments (we now state this explicitly in the Discussion). Regarding our predictive capacity, in the current revision we tried to quantify it by splitting observables into a test set and a training set (with ratio $1:9$), showing that there is a value of the hyper-parameter ruling the prior and/or regularizer where observables are predicted with a reduced average square residue of $\chi^2 \simeq 0.8$ (see Fig. 1 below, as well as Fig. 4, panel B, red markers in the main text). We furthermore included a new Section S11 in the Supporting Information devoted to the discussion of several variants of the model (non-interacting, fitting only average and variance and trying to predict correlations, neglecting background lactate) and their capacity to fit and predict the data. We hope this provides a more clear picture of the performance of our model.

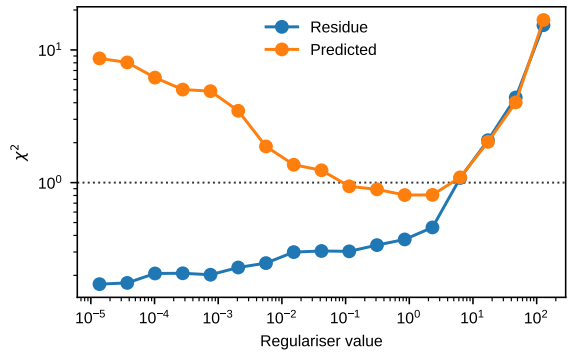


Figure 1: Reduced $\chi^2$ (average square residue) for the fitted and predicted data as a function of the hyperparameter $\gamma$

1.3 *The abstract ends with:*

> *In summary, our findings suggest that, rather than deriving from multiple independent cell-autonomous processes, environmental control is an emergent feature of multi-cellular systems.*
>
> *There are similar grand-sounding statements throughout the manuscript often about "the breakdown of coordination". As far as I understand the work, all of the results are explained by the meanfield model, which assumes that each cell is independent except they all have access to the same pool of lactate in their environment. That is each cell has the same "strategy" and sees the same environment and these two factors are the only sources of collective behavior. I feel that such scenarios should be described in much less grandiose terms. For example, stochastic secretion of lactate is limited by negative feedback (cells consuming excess lactate) until majority of cells in the population switches to lactate production.*

The necessary premise is that, with the new inference pipeline, we now put a much greater emphasis on the dynamical, off-equilibrium nature of the system. In light of this, interpretations based on the mean-field model alone, while conceptually correct, are quantitatively incorrect. We have therefore substantially revised all the statements to which the Reviewer is referring.

Nevertheless, the new results we present significantly strengthen the idea that the full set of empirical data at our disposal cannot be retrieved through cell-autonomous models that ignore cell-to-cell couplings (see Sec. S11, Supporting Information). More specifically, they cannot be retrieved by models of cells that independently maximize an objective function.

Concerning 'grandiose statements', we fully share the Reviewer's idea that such statements should be avoided. However, the words 'breakdown/failure of coordination' are, in our view, a factual description of the results *of the mean-field model*. In the balanced phase, cells achieve near-zero mean lactate output by correlating their metabolisms. By contrast, in the Warburg phase such a coordination does not occur and lactate spills over in the medium. At the quantitative level, the breakdown of coordination can be seen in the sudden increase of pairwise correlations that occurs in correspondence of the overflow threshold, see Figure 3A. The new section S9 of the Supporting Information now provides further evidence on inter-cellular correlations.

For sakes of simplicity, we report below (Figure 2) Fig S9 from the Supporting Information. The figure shows the standard deviation of the lactate flux for a single cell ($\sigma_{u_L}$, rescaled by $1/\sqrt{N}$), and the standard deviation of the mean across cells ($\sigma_{\bar{u}_L}$), as functions of $\beta_G$ (for fixed $\beta_O$). The overflow transition takes place at $\beta_{G,c} \simeq -1.5$: for $\beta_G > \beta_{G,c}$, we have that $\sigma_{\bar{u}_L} \simeq \sigma_{u_L}/\sqrt{N}$, implying lack of correlations; for $\beta_G < \beta_{G,c}$, instead, $\sigma_{\bar{u}_L}$ becomes very small, implying the presence of negative correlations among cellular lactate

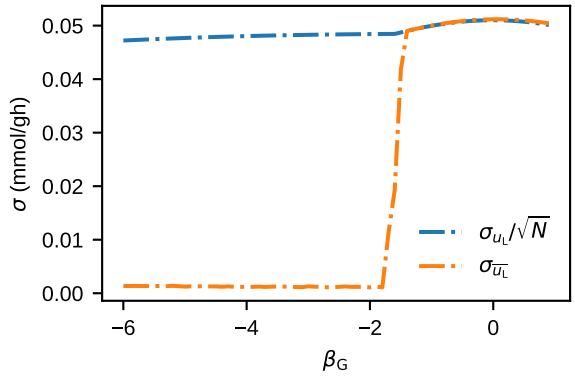


Figure 2: Standard deviation of the lactate flux for a single cell $\sigma_{u_L}$, rescaled by $1/\sqrt{N}$, and standard deviation of the mean across cells $\sigma_{\bar{u}_L}$, as functions of $\beta_G$ for fixed $\beta_O = 1$ from mean field calculations.

fluxes, described by

$$\sigma^2_{\bar{u}_L} = \frac{\sigma^2_{u_L}}{N} + \frac{1}{N^2}\sum_{i\neq j}\langle((u_L^{(i)} - \langle u_L\rangle)(u_L^{(j)} - \langle u_L\rangle)\rangle \quad . \tag{1}$$

It is however worth stressing again that, with the more dynamical point of view we now adopt in our inference pipeline, this scenario only provides a qualitative reference frame for understanding results.

On the other hand, the Reviewer is completely correct in noting that results would change upon adding more/different ingredients to the (minimal) metabolic model we employ for individual cells. We chose to adopt a parsimonious approach to modeling in the hope of highlighting the key mechanisms behind the biological phenomenon (specifically, the role of interactions). But it would definitely be important to look at these results through the lens of more realistic and possibly large-scale models. (This will however require the use of different computational setups with more powerful algorithms and statistical inference tools than those we employed.)

Concerning the specific example made by the Reviewer ("*stochastic secretion of lactate is limited by negative feedback*"), we fully agree that it would most likely have a significant effect especially on the dynamics of the system, e.g. on equilibration times. However, it is still not completely clear to us what would drive *the majority of cells to switch to lactate production*, unless some *ad hoc* field forcing cells in that direction is included (e.g. the maximization of some objective function).

1.4 *I also have some concerns about the simulations underlying the reported results.*

(A) *I don't think the spatial distribution of cells adds much to the study because it gives the same results as the meanfield model and depends on numerous additional and often unrealistic assumptions. The latter include diffusion of lactate, but not glucose, 3D diffusion of lactate, but 2D arrangement of cells, no temporal dynamics, etc.*

We again thank the Reviewer for a very useful comment. The actual role played by the spatial distribution of cells in the overall phenomenology we observe is indeed a very important point. In line with the Reviewer's comment, the diffusive constraint as implemented in the mean-field model, which effectively washes the spatial organization of cells away, is indeed capable of reproducing some of the empirical evidence we rely upon. However, other features we can extract from data (like nearest-neighbour correlations) are not reproduced by that constraint alone, most notably when the lactate background term is properly accounted for. In order to retrieve these features

in our inference pipeline we must include an additional term that effectively amounts to a direct cell-to-cell interaction. From a physical viewpoint, this term can be thought to represent spatial details of the diffusion constraints that get discarded in our highly simplified mean-field theory but are essential to fully represent our dataset. This however suggests that the spatial organization of cells is rather important. In this respect, it would in our view be highly desirable to have a tractable dynamical model of the population that is capable of accounting for it.

At any rate, as a consequence of all this, the spatial model we discuss plays, in our view, a major role in our work by providing two unique types of results. Firstly, it validates, in a more realistic setting than the mean-field model, the idea that diffusion-limited exchanges are the main drivers of inter-cellular interactions. Secondly, as we noted above, only the spatial model can yield a wide range of testable predictions (e.g. for the distribution of lactate fluxes across the population, for the cell-to-cell correlations in metabolic activity, or for oxygen usage/mitochondrial saturation patterns).

To summarize this part: unless one can devise a finite-dimensional model that is solvable, in our view *both* spatial models and their simplest tractable mean-field approximations are useful to get a clear picture of how factors like spatial organization, interactions, diffusion, boundary conditions etc. affect the behaviour of a system of interacting units.

Regarding our choices on dimensionality, they were tailored on the basis of how the experiments we considered in the final part of the paper were conducted. In particular,

- Experiments were performed with a standard in vitro culture where cells adhere to a substrate, effectively leading to a two-dimensional organization. Molecules, on the other hand, were free to diffuse in the $z$-direction, since they are much smaller. This is why we assume the stationary density to be described by the solution of the three-dimensional Laplace equation (Supporting Information, Sec. S2). Truly 3D cultures endowed with microenvironment-sensing nanofibers are only starting to explored [Siciliano et al, A 3D Pancreatic Cancer Model with Integrated Optical Sensors for Noninvasive Metabolism Monitoring and Drug Screening. Adv Healthcare Mat (2024)]. The model will certainly have to be updated in order to describe these systems.

- In addition, experiments were performed in a DMEM medium with glucose concentration $c_G = 25$ mM. Taking $D_G = 600\,\mu\text{m}^2/s$ (diffusion constant of glucose), $R = 10\,\mu$m (cell radius), $M = 1$ ng (cell mass) and $K \equiv NR/L \simeq 40$ (crowding factor in the mean-field approximation, with $N$ the number of cells and $L$ the linear size of the system), this implies a diffusion-limited glucose uptake bound given by $4\pi c_G D_G R/(Km) \sim 170$ mmol/gh. While a rough estimate, this value exceeds the capacity of glucose transporters (about 1 mmol/gh even at extreme cell densities) by two orders of magnitude. In other words, at the glucose concentrations employed in the experiments, glucose diffusion can be safely neglected. A similar situation holds for oxygen, where the figures are 6 mmol/gh and 3 mmol/gh for the diffusion-limited oxygen uptake and mitchocondrial maximum oxidative flux, respectively. The two bounds are nearer wrt the case of glucose and we have performed simulations with and without intercellular diffusion constraints for oxygen to confirm that indeed they play no role for the cases we analyzed in this paper (but they could upon eg doubling cell density).

In summary, we believe that our choices regarding the diffusion of compounds and the dimensionality of the system are justified. We have now stressed this in the revised version of the article, specifically in a new section of the Supporting Information (Sec. S8) where intercellular diffusion constraints of oxygen and glucose are analyzed in detail. Such details were indeed missing in the original submission, and we thank the Reviewer for raising these points.

Coming finally to the lack of temporal dynamics in our original study, we completely agree with the Reviewer that the dynamical aspect is crucial to understand how the population organizes and our original submission was over-simplifying in this respect and, as said above, we have now improved our modeling approach and inference pipeline to account for this specifically. More precisely, we considered the explicit role of the time-dependent (increasing) background lactate level that is observed experimentally [Onesto et al, Probing Single-Cell Fermentation Fluxes and Exchange Networks via pH-Sensing Hybrid Nanofibers, ACS Nano 2023]. We first extended our mean-field model to account for it, showing (i) that, as the background level changes, the phase transition point shifts toward more negative values of $\beta_G$ and $\beta_O$, and (ii) that the balanced phase expectedly disappears for large enough backgrounds. We then included the background term in the inference pipeline, effectively accounting for a dynamical forcing in the reconstruction of the dynamics of the population in the $(\beta_O, \beta_G)$ space. The new inferred experimental trajectory which yields the best representation of our data is qualitatively similar to the previous one but now stays fully within what would be a Warburg phase at equilibrium, due to the shifting of the critical line. These new results are summarized in Figure 4 of the main text (and in the discussion around it), as well as in Sec S11 of the Supporting Information.

(B) *One of the key assumptions is that all feasible points in $\mathcal{F}_N$ are equally accessible, i.e. have the same weight up to the Boltzmann factor. It is not clear how one would obtain such joint distribution from each cell following a Boltzmann distribution (perhaps dependent on the lactate concentration in the medium). My question is whether this assumption adds an "invisible hand" that influences the dynamics in ways beyond what could be achieved by independent cells in common environment.*

We thank the Reviewer for raising this point. The short answer is that yes, by describing the population through a joint distribution that accounts for the possibility of exchanges we are indeed expanding the capabilities of individual cells beyond what they would be allowed to do within a cell-independent model (assuming no external source of lactate). However, it is important to stress that, in our theoretical setup, that is both in our analysis of the finite-dimensional model and in the mean-field model, the Boltzmann distribution only serves the purpose of providing a tunable way to explore the feasible space (while allowing for exchanges). In other words, by modifying $\beta_G$ and $\beta_O$ we simply focus on different parts of $\mathcal{F}_N$, whereby the difference lies in how much glucose and oxygen cells intake (on average). In this way, we can extract 'typical properties' of cell populations described by different values of $\beta_G$ and $\beta_O$. (Note that no underlying dynamics is assumed here.) $\beta_G$ and $\beta_O$ are merely convenient parameters in our view, because glucose and oxygen are the key nutrients externally supplied in experiments. (One could equally well use 'inverse temperatures' related to lactate and glucose, for instance, but, since lactate is only endogenously produced, the connection to experiments would be less straightforward in our view.) In other words, by changing the Boltzmann factors that appear in our models we are not implying that cells necessarily have agency or are trying to optimize something (with some 'thermal fluctuations').

It then follows that the values of $\beta_G$ and $\beta_O$ we obtain from our inference protocol are those that provide the best description of the data, under the assumption that data are described by our probabilistic model and the chosen empirical constraints. In this respect, the dynamical trajectory we derive (corresponding to how the inferred $\beta$s change in time) simply provides a reduced, compact representation of the complex, high-dimensional trajectory that the population performs in the feasible space. In turn, having a tractable model that can distinguish different regimes in the low dimensional space of $\beta$s may give us some ability to interpret this compact representation at a deeper physical level. We have now tried to clarify these points, both in the Introduction and in the Discussion.

Regarding the last part of the Reviewer's comment ("*whether this (Boltzmann) assumption adds an "invisible hand" that influences the dynamics in ways beyond what could be achieved by independent cells in common environment*"), we would like to stress that the Boltzmann weights we are considering do not, by themselves, couple cells, since the Lagrange parameters $\beta_O$ and $\beta_G$ multiply the linear sum of oxygen and glucose fluxes respectively. This leads to factorization, as in

$$e^{\beta_G \sum_i u_{G,i}} = \prod_i e^{\beta_G u_{G,i}} \quad . \tag{2}$$

In other terms, without additional constraints, this model treats cells as independent. The source of the coupling (and therefore of the fact that extra states become accessible to cells that are not accessible when they are independent) comes from the constraints induced by diffusion, which define linear half-spaces that cut across the single cell metabolic spaces so that the total space cannot be factorized (pretty much in the same way in which a simplex is different from an hypercube).

(C) *Another assumption is the choice of $h(u)$. I think some previous works using this formalism assumed that $h(u)$ is the growth rate rather than the incoming fluxes. Would this significantly change the results?*

The Reviewer is referring (we believe) to previous work by some of the authors on Maximum Entropy modeling of bacterial populations and subsequent generalizations and extensions [see e.g. Physical biology 13:036005 (2016); Nature communications 9:2988 (2018); Journal of Mathematical Biology 80:2395 (2020); Iscience 25:105450 (2022)]. These studies aimed in essence at reproducing the distribution of single-cell growth rates in *E. coli* cultures in steady growth conditions, specifically to test the hypothesis that the empirical variability is the largest possible given the mean growth rate. In that case, the $\beta$ factor appearing in the Boltzmann distribution was directly coupled to the growth rate. We don't think the same framework can be applied to the system we consider here for two main reasons.

(a) The populations we analyzed here are in the adaptation (lag) phase, as opposed to the exponential phase that is normally the focus in studies of microbes. It is not obvious, in our view, that the growth rate (or one of its proxies, such as the ATP synthesis rate) is a relevant control parameter in the lag phase (especially for the kinds of mammalian cell systems on which we concentrate).

(b) In previous studies, cell-to-cell interactions and exchanges were ignored, as cells were assumed to be independent. Such an assumption is reasonable for bacteria in the exponential phase. It is not clear to us that the same holds during lag phases (bacterial or otherwise).

We have now attempted to be more clear about this specific choice in the main text, before Eq. 13. We hope this clarifies our motivations.

The Reviewer's remark is however very interesting when brought beyond the study we present, as constraint-based modeling is particularly successful for microbial cultures and not much is known about how microbes adapt to a medium or crosstalk. The approach we use in the present work can in principle be extended to such cases upon suitably modeling growth and division. We refer to our reply to point 2.3 of Reviewer 2 for further details.

(D) *Finally, the parameterization in terms of $\beta$ has two effects. The first effect is that it switches the corresponding flux from minimal to maximal. The switching point is around $\beta = 0$. At the same time, $\beta$ controls the fluctuations, which are maximal around $\beta = 0$ as well. Thus when the authors report large fluctuations near the transition between coordinated and overflowing states, they potentially conflate two phenomena: the*

*increase of fluctuations near a phase transition due to collective effects and the increase of microscopic fluctuations driven by the choice of the parameterization in terms of $\beta$. In the physics language, this would correspond to choosing a temperature-dependent coupling in the Ising model such that $J$ has a minimum at the critical temperature.*

Hoping we understand the Reviewer's point correctly, there are in our view some subtleties to consider. First, the Reviewer is totally correct in stating that fluctuations are maximal when $\beta_G = \beta_O = 0$, i.e. when the population can sample the feasible space uniformly (one can see this directly in Fig. 3A). However the transition in the mean-field model does *not* generically occur at or close to the point $\beta_G = \beta_O = 0$ this is also seen in Fig. 3, both in the critical line and in terms of the separation between the point where the variance peaks and that where the variance changes discontinuously). So if we observe any kind of singularity around a critical line away from $\beta = 0$ we are genuinely observing a change in collective behavior that accompanies phase transitions. In our case, at odds with the Ising model, we do not have a peak/divergence of specific heat, susceptibility etc but we have discontinuous jump for intercellular correlations. The mean-field model indeed confirms this picture quantitatively, both in the original version without lactate background and in the revised one that accounts for it. (Note however that the time-dependent trajectory in the space of $\beta$ inferred in this revision now provides a different picture for what concerns the self-organized criticality of the population.)

About the Ising model: to our understanding, temperature (i.e. $\beta$) does not really play a role when one tries to infer, say, the couplings $J_{ij}$ from a set of spin configurations, since they can always be re-scaled by whatever $\beta$ factor one puts in front of them. So inference is effectively always made at "inverse temperature $\beta = 1$". Again as far as we understand, the reason why the inverse temperature $\beta = 1$ appears critical when one studies the Ising model built upon the inferred couplings is that that temperature is not just any temperature: it's the one at which couplings were inferred. This partly explains why inferred Ising models tend to look 'critical'. (This is however a very subtle theoretical issue [e.g. On the criticality of inferred models, J. Stat. Mech. (2011) P10012] that goes way beyond our present work.)

More broadly, though, we think that the Ising model is not necessarily a good paradigm for the type of problem we study here, because

(a) contrary to Ising models in which a large number of parameters is inferred (usually $\sim N^2$ for a system of $N$ spins), we only infer 2 or 3 parameters for a system of $N$ cells, depending on whether nearest-neighbour correlations are accounted for in the inference protocol. That is, in Ising model terms, what we infer is $\beta$, not the couplings. In this respect, lessons learned from the inference of couplings do not easily transfer here, in our view.

(b) The way in which fluctuations are related to $\beta$ in our system is not straightforward as in the Ising model because of the presence of the diffusive constraint.

We have now further stressed this difference in the Discussion section of the main text.

1.5 *In Fig. 2, it would be interesting to see how the average $u_L$ behaves as a function of $\beta$ and $u_G$ for a single cell bathed in unlimited lactate. In particular, I wonder how the location and steepness of the collective transition differ from that in a single cell (presumably the single cell would also have a region of $u_L = 0$ and $u_L > 0$).*

We thank the Reviewer for this remark. The mean lactate efflux for an isolated single cell and for a system of 150 cells are reported in Fig. 3 below, as functions of $\beta_G$, respectively in presence of an unlimited exogenous supply of lactate (left panel) and in absence of exogenous lactate (right panel). Expectedly, the profiles obtained for a single cell and for the population coincide when they're bathed in infinite lactate, because in this condition

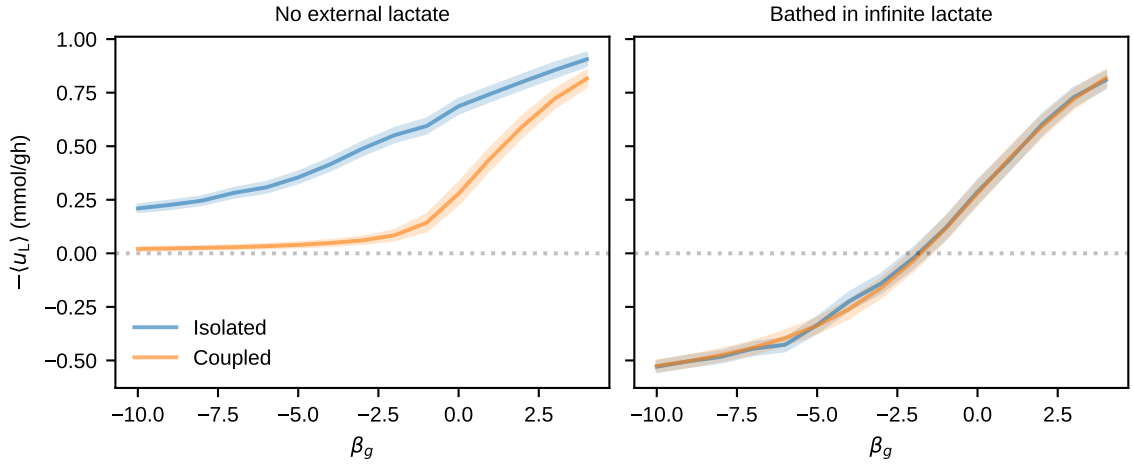


Figure 3: Average lactate flux as a function of $\beta_G$ for $\beta_O = -1$ for a single cell and for $N = 150$ interacting cells without endogenous lactate and with unlimited external lactate, from model simulations.

the diffusion constraint is effectively irrelevant. On the other hand, in absence of external lactate the two situations diverge significantly. While a single cell consistently displays a positive lactate efflux, populations can drastically reduce lactate spillover into the medium when $\beta_G$ is sufficiently small. The resulting behaviour is strongly reminiscent of an emergent phenomenon (a phase transition from a regime with zero lactate efflux to one with non-zero lactate efflux). A single cell cannot give rise to this kind of phenomenology unless a sharp change in the way it uses metabolic reactions is built-in from the outset in the model (e.g. by imposing additional constraints that force a switch from, say, lactate excretion to lactate import; or by imposing that the cell is optimizing some objective function whose maximum jumps discontinuously as one changes some parameter; this is clearly not our case as we are making no such assumptions). More precisely, transitions in the behaviour of any statistical feature of a flux (mean value, variance, etc.) versus $\beta$ imply that a derivative with respect to $\beta$ of the logarithm of the partition function of the system (which is used to generate the statistics of fluxes) is non-analytic at some $\beta$ (the 'critical value'). Strictly speaking, such non-analyticities can only occur when cells are interacting and their number $N \to \infty$. This fact is the very essence of collective phenomena. On the other hand, finite systems of interacting cells can show signatures of criticality, which become more and more pronounced as the number of cells increases. And indeed our simulation results for the spatial model show that, when $N$ is sufficiently large and cells are interacting, the system does display signatures of the phase transition, which is unveiled in the highly stylized mean-field model where the limit $N \to \infty$ is explicitly taken.

1.6 *I wonder if the deviations between the meanfield and the spatial model are due to the fact of finite $N$ rather than the effects of space.*

The Reviewer is raising a very interesting (albeit very technical, to our understanding) comment. If the difference was only due to the fact that $N$ is finite in the spatial model, then the spatial and mean-field models should coincide in the limit $N \to \infty$. This convergence is certainly possible, but it usually requires the dimension of the spatial model to be large enough (more precisely, larger than a model-specific 'critical dimension'). While continuous models like the one we are dealing with tend to have relatively large critical dimensions, we could not find a path to conclusively evaluating the critical dimension of the model we are dealing with. In principle, one could see whether the behaviour of the spatial model in the limit $N \to \infty$ tends to that of the mean-field model by using *ad hoc* computational techniques like finite-size scaling [Privman (ed), Finite size scaling and numerical simulation

of statistical systems. World Scientific (1990)]. Such a study would certainly be interesting, but it requires extensive simulations of the spatial model with increasing number of cells (in terms of orders of magnitude) and multiple realizations of the spatial arrangements of cells. This is beyond our possibilities at present. However, the mean field approximation and the spatial model differ substantially in the nearest-neighbor correlations they generate, the spatial model showing a correlation pattern more similar to that observed experimentally. Although clearly neither conclusive nor rigorous, these results seem to indicate that the spatial arrangement does matter in 2D. We have now reported them in a new Sec. S9.3 of the Supporting Information (see also Figure 4 below).

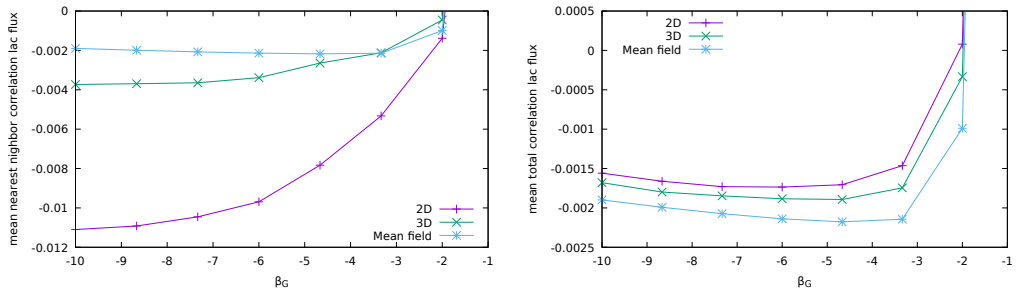


Figure 4: Average lactate flux nearest neighbor (left) and total (right) correlations as a function of $\beta_G$ at fixed $\beta_O = 1$ for systems of different dimensionality (2D, 3D and fully connected mean-field approximation).

1.7 *I would double check the caption of Fig. 1B; I think some points are not correctly labeled.*

We thank the Reviewer for the remark. We have double checked the caption and it seems correct.

1.8 *The application of theory to cancer data has several problems.*

(A) *The first one is that the model assumed a steady state, but the cancer data has a very strong time-dependence. I don't think 1 hour time scare is enough for the equilibration of nutrients and metabolic fluxes.*

We thank the Reviewer for raising this important point. Our stationarity assumption is based on the following estimates. The diffusion coefficient of lactate is roughly $D_L \simeq 700\,\mu\text{m}^2/\text{s}$. As the linear size of the observed system is $L = 0.5\,\text{mm}$, the timescale for diffusional relaxation of lactate is about $L^2/D \simeq 6\,\text{min} \ll 1\,\text{h}$. A very similar picture holds for glucose ($D_G \simeq 600\,\mu\text{m}^2/\text{s}$). Oxygen diffuses much faster than both. In other terms, nutrient levels are highly likely to equilibrate on timescales much shorter than experimental ones. For what concerns the relaxation timescales of cellular metabolites and metabolic fluxes, turnover times range from minutes (for amminoacids) to seconds (ATP) to milliseconds (nicotinammidic cofactors) [Milo & Phillips, Cell biology by the numbers. Garland Science (2015). Note that the book reports estimates obtained for *E coli*. Still, even if timescales for mammalian cell cultures would vary by a factor of 10 compared to bacterial timescales, we would be well below 1 h.]. We have now added these considerations to the main text to justify our steady state assumption, specifically in the revised section "Exchange coupling and multi-cellular flux space" before and after Eqs 6 and 7. We hope this clarifies our setup.

(B) *Moreover, the environmental concentration of lactate goes down, so there is a net consumption of lactate, which was forbidden in the theory.*

We apologize for the confusion. In the previous version of the manuscript we focused Figure 4 on the *change in lactate spatial gradient*, neglecting to show the background lactate level that comes from the accumulation of lactate over time. This gave the wrong impression that the average lactate level is decreasing while in fact it stays roughly constant for over half of the experiment's duration (at $c_L \simeq 10\,\mu$M) and then increases (rapidly over the last hour, up to $c_L \simeq 40\,\mu$M). This was reported in [Onesto et al., Probing single-cell fermentation fluxes and exchange networks via pH-sensing hybrid nanofibers. ACS Nano (2023)]. In turn, average fluxes, albeit decreasing in magnitude, are always in the secreting direction (i.e. negative in our convention). These patterns are showin in Figure 5 below, which displays the experimental trend measured independently by a Seahorse assay using lactate dehydrogenase and by integrating over the average lactate flux.

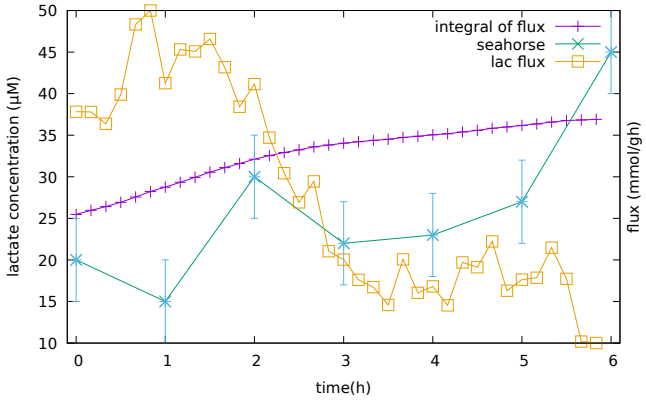


Figure 5: Experimental lactate concentration as function of time, obtained from a lactate dehydrogenase assay (circles) and by integrating over average cell lactate flux (crosses). Also shown is the average lactate flux. (squares). Data from [Onesto et al., Probing single-cell fermentation fluxes and exchange networks via pH-sensing hybrid nanofibers. ACS Nano (2023)].

The reason we omitted to show the background lactate in Fig. 4 was that, for $c_L \simeq 0 - 10\,\mu$M, the factor $4\pi c_L D_L R/(Km)$ regulating the diffusion constraint is $\simeq 0.1$ mmol/gh, which should be compared with the limit imposed by oxidative capacity ($\simeq 1$ mmol/gh). However, during this revision (triggered by Reviewer comments), we realized that things change when $c_L \simeq 35 - 50\,\mu$M (i.e. in the last hour of the experiment). As explained above, we have therefore included the background lactate term for good in our analysis, fully considering its empirical time dependence. This has in turn lead to important changes both in the nature of the models we must consider (which should be dynamical and out-of-equilibrium) and in the inference protocol. Such changes have been included in the manuscript, both in the main text and in the Supporting Information (most notably in Sec. S11), while Figures 4 and 5 have been entirely revised. Furthermore, a new section (S7) has been added to the Supporting Information, devoted specifically to the effects induced in the model by background lactate accumulation. Again, we apologize for the confusion.

Related to the above, we have also included the background term in the diffusion constraint appearing in the mean-field model. Intuitively, once all parameters are fixed (e.g. cell density, cell radius etc.), one would expect there to be an external lactate level for which the Warburg regime becomes unavoidable. We indeed found that the phase transition will disappears when the background lactate level exceeds the value

$$c_{L,b,th} \simeq 100\,\mu\text{M} \quad . \tag{3}$$

The trend seen for increasing values of the background lactate level is illustrated in Fig. 6 below, where the overflow curve is displayed for several values of $c_{L,b}$.

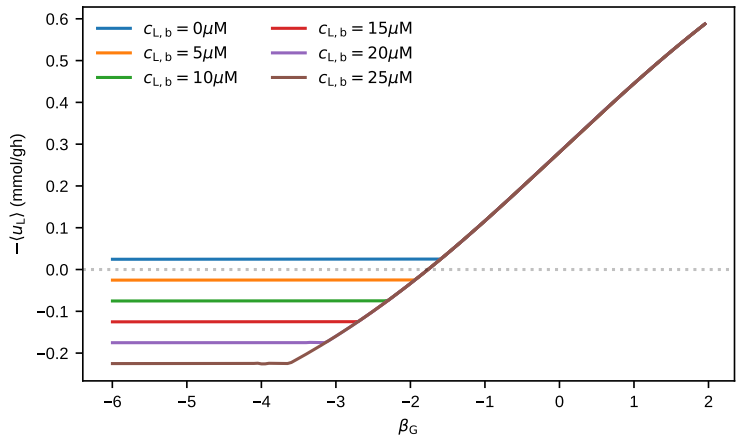


Figure 6: Average lactate flux as a function of $\beta_G$ at fixed $\beta_O = 1$ for several $c_{L,b}$ (in $\mu$M) from mean field calculations.

(C) *Secondly, I don't understand why the accumulation of data on the boundary between the regions is surprising. In Fig. 4D, the other side of the boundary is inaccessible (net consumption of lactate). So a simple explanation would be that the cells just feel/grow best without excreting too much lactate. The accumulation near boundary in Fig. 4C could be an artifact of a very noisy experimental data (this noise could be measurement noise not the real heterogeneity) and the fact that the theory has the greatest noise near the transition. So in order to match the noise in the data, the parameters need to be near the transition.*

The Reviewer is absolutely correct, the mechanism they propose can indeed explain why inferred models tend to be critical [see e.g. On the criticality of inferred models, J. Stat. Mech. (2011) P10012]. We however note that, with the new inference results, such an accumulation no longer occurs (except at initial times), since the system is now dynamical and the critical line corresponding to different time frames of the experiment shift down in the $(\beta_O, \beta_G)$ plane. Proceeding from the Reviewer's observation, this testifies, we believe, to the fact that there is no overfitting in our results (after all, as mentioned above, we need to infer only two parameters in order to describe the whole population). Unfortunately, in the new dynamical setup it is much harder to interpret the trajectory of the system using the critical line(s) coming from the mean-field model as a reference. On the other hand, in the revised version of the manuscript we can report the error bars on the inferred $\beta_G$ and $\beta_O$ (see Figure 4 in the main text and Supporting Information, Sec. S11).

(D) *Thirdly, I did not understand the reasoning behind Fig. 5. It seems that the authors say that low oxygen uptake results in high oxygen concentration, which is totally logical from the reactions. However, this means that the cell cannot have both low oxygen uptake and low oxygen concentration, which is the true state of hypoxia. I think many of the claims are based on the fact that the media is not hypoxic when the cells take up little oxygen, but such cells grow "hypoxically"!*

We thank the Reviewer for this comment. In short, Figure 5 presents a set of predictions that can be made on the basis of our theoretical analysis, which concern oxygen usage by cells. More precisely, inference results suggest that, for the specific population we consider (or most notably for that specific cell density), no local hypoxia occurs. This rules out one of the known triggers of the Warburg effect [Chen et al, Hypoxic microenvironment in cancer: molecular mechanisms and therapeutic interventions, Signal Transduction and Targeted Therapy (2023)]. Indeed, inferred oxygen

uptake levels are sustained, even in comparison with previous experiments [Wagner et al, The rate of oxygen utilization by cells. Free Radic Biol Med (2011)]. So, in the population we consider, we find (weak) acidification accompanied by sustained oxidative fluxes and no local hypoxia. We also find that cells saturating their oxidative capacity (another possible cause of the Warburg effect) constitute a very modest fraction of the population (Fig. 5C of the main text). In addition, those that saturate do so for both glucose and lactate with a slight preference for the latter, while only in the former case this leads to overflow. All in all, the picture that emerges is that neither local hypoxia nor saturated oxidative capacity appear to play a relevant role for the onset of the Warburg effect in our case study. This is, in our view, further indication that lactate spillover in our population is connected to the pattern of correlations of single-cell metabolic activities. Lack of correlations is the major cause for acidification.

We clearly cannot exclude that for different experimental settings (e.g. different cells densities, population ratios of tumor cells versus fibroblasts, etc.) the aforementioned factors could be modulated differently and therefore become highly relevant. If so our framework could potentially accommodate for such effects.

We have now attempted to better clarify the scenario underpinned by Fig. 5 by streamlining the main text surrounding it. We also added a new section S8 to the Supporting Information, focused on the oxygen diffusion limitation constraints, explaining why they do not matter for the conditions we analyzed. For sakes of simplicity, we report Figure S6 in Fig 7 below. One sees results from simulations of the spatial model at different background oxygen levels. Specifically, the average oxygen flux is plotted as a function of $\beta_G$ (at fixed $\beta_O = 1$) for $c_{O,b} \to \infty$ (i.e. not including the constraints), $c_{O,b} = 250\,\mu$M (room conditions as in experiments), and for different degrees of hypoxia ($c_{O,b} = 150,\ 80,\ 60\,\mu$M). As expected, hypoxia begins to influence results significantly when $c_{O,b}$ falls below $100\mu$M roughly.

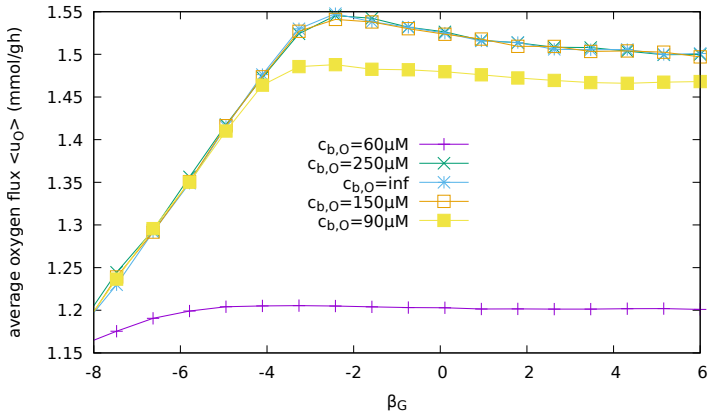


Figure 7: Average oxygen flux as a function of $\beta_G$ at fixed $\beta_O = 1$ for several $c_{O,b}$ (in $\mu$M) from simulations.

We stress again that results displayed in Figure 5 of the main text are *predictions* of our model which can be experimentally validated, for instance by oxygen-sensing microfibers. Experiments in this directions are in progress [see e.g. Grasso, et al, Highly sensitive ratiometric fluorescent fiber matrices for oxygen sensing with micrometer spatial resolution, Bio-Design and Manufacturing (2024)].

1.9 *Finally, any claim about collective effects should be made only after comparing the behavior of a single cell and a collection of cells. The single cell model should allow for a reservoir of lactate. That is at a given time step the cell can either uptake lactate from the reservoir*

*(if it has some) or excrete some lactate that is stored in the reservoir. One can then plot the average lactate concentration in the reservoir as a function of N and based on that talk about collective behavior. It seems that at least some of the observations are due to the fact that there is a reservoir in the environment not some intricate coordination among the cells.*

We are very grateful towards the Reviewer for pointing us in this direction. As we have shown above (Figure 3 in this reply), a single cell in a lactate reservoir in general gives rise to markedly different phenomenology compared to a population of interacting cells: a model formed by non-interacting, uncoupled cells by construction cannot reproduce the negative correlations we observe in data. This is explained in detail in the new section S9.3 of the Supporting Information along the following lines.

For $N$ cells in a given random spatial configuration (from experiments or simulations) we define average nearest neighbor inter-cellular lactate flux correlations by the expectation value of

$$C_{n.n.} = \frac{1}{N} \sum_i u_L^{(i)} u_L^{(n(i))} \tag{4}$$

where $n(i)$ is the index of the first neighbor of the cell $i$, based on absolute distance. In the absence of pairwise correlations, the expectation value of $C_{n.n.}$ can be identified with the square of the average lactate flux since

$$\langle C_{n.n.} \rangle_{unc} = \frac{1}{N} \sum_i \left\langle u_L^{(i)} u_L^{(n(i))} \right\rangle = \frac{1}{N} \sum_i \left\langle u_L^{(i)} \right\rangle \left\langle u_L^{(n(i))} \right\rangle = \langle u_L \rangle^2 . \tag{5}$$

As shown in Figure 8, left, this is reproduced by simulations of isolated single cells without exogenous lactate, as considered in section S7. On the other hand, the coupled model displays

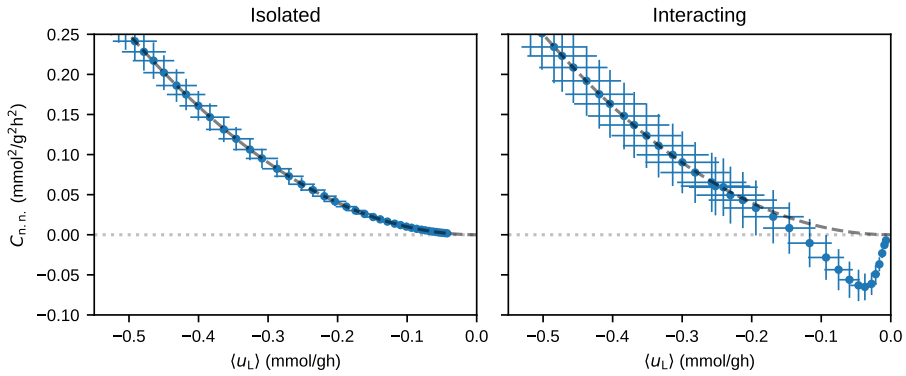


Figure 8: Left: Nearest neighbor correlations vs average flux for a model of isolated single cells verifies a simple scaling relation. Right: correlations vs the average flux for the coupled model violate the simple scaling relation. From simulations of the spatial model with $N = 150$ cells performed via Monte-Carlo sampling, sweep in $\beta_G$ ($\beta_O = 1.$)

negative nearest neighbor correlations in the balanced phase, as displayed in Figure 8, right. Moreover, a non-interacting model cannot even reproduce average and fluctuations if we exclude correlations from the observables to fit (see Fig 9 below). By contrast, the interacting model can generate negative inter-cellular correlations, as we now report in Sec. S11 of the Supporting Information as well as in Fig. 4 of the main text.

A more detailed analysis confirms this scenario. Fig. 10 below reports the scaling of fluctuations with respect to the mean for both the non-interacting and interacting models. One clearly sees that the observed pattern (orange markers) lies outside the range of patterns that can be generated by a non-interacting mode, whereas an interacting model can reproduce it. This is now reported in the new Section S11 of the Supporting Information.

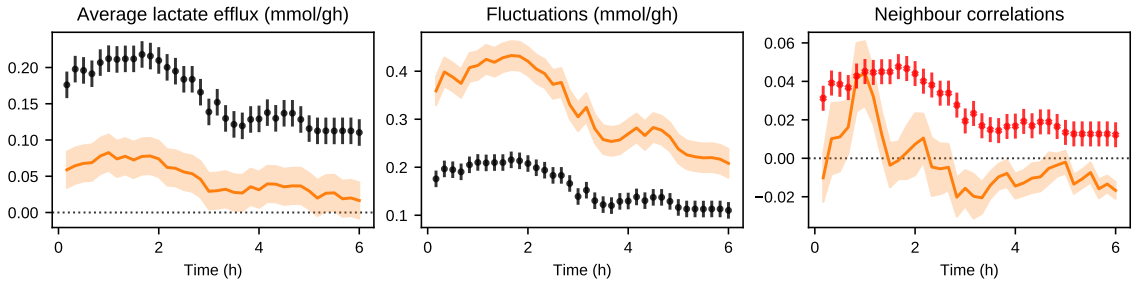


Figure 9: Fit of average and standard deviation of experimental lactate fluxes (orange line) with a non-interacting model (black dots).

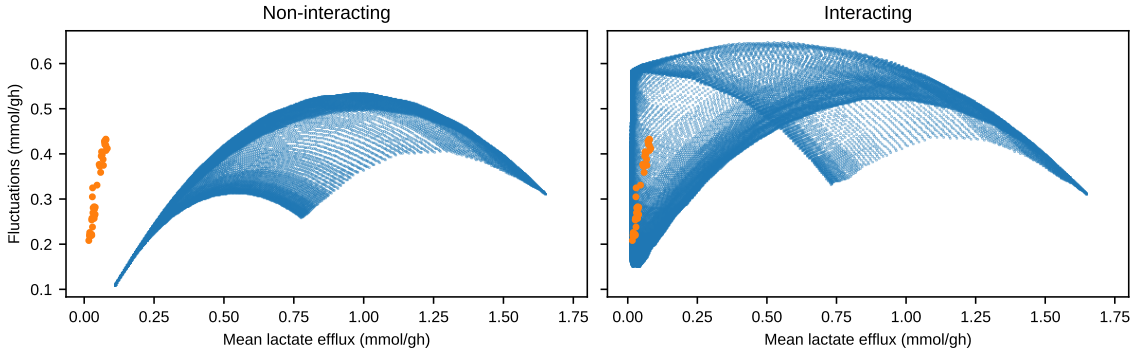


Figure 10: Scaling of the standard deviation vs average lactate flux for experimental data (orange markers) vs models (blue markers): non-interacting model (left) versus interacting model (right). Each marker corresponds to a pair of values $(\beta_O, \beta_G)$.

We must conclude that, ultimately, the ingredient that plays the key role in generating the scenario we uncover is represented by cell-to-cell correlations in metabolic activities.

On the other hand, as the Reviewer suspected, the transition in the model (as well as any collective behavior triggered by lactate exchange) shifts when the external lactate level becomes high and cells must take in lactate to correct the imbalance. Higher concentrations are indeed achieved in the final phase of the experiments we considered, and we have now corrected our inference and modeling approaches to account for them.

Again, we are very grateful to the Reviewer for multiple useful comments and suggestions.

## Reply to Reviewer 2

*In this paper, the authors study the phenomenon of overflow metabolism, a process that occurs in microbial communities as well as tumor microenvironments. A statistical and analytical framework is used to show that a phase transition occurs between a balanced state with low concentrations of potentially toxic overflow products, and an unbalanced overflow state marked by high heterogeneity of flux across cells. They then show that in an experimental co-culture of cancer cells and fibroblasts, the dynamics map to the critical line separating these phases. They argue that these results show that the state of the environment is an emergent effect of inter-cellular interactions.*

*The last point, about the emergent effects, is the most unclear of the paper's main claims. Overall, however, the paper is well-written and clear, with convincing explanations of the methods and results. The topic will be broadly interesting to researchers studying various systems. I have some suggestions for clarifying a few points, and for describing the results to those studying other systems.*

We thank the reviewer for their very positive comments on our work. Regarding clarity on emergent behavior and the paper's aims, we refer them to our response to Reviewer 1's general point (at the beginning of our reply), where we describe how we have considerably improved the description of both. In agreement with the referee's concerns, we however decided to substitute the term "emergent behavior" with "metabolic coordination" in the title, that we believe address now more satisfactorily the main content of the work.

2.1 *First, a better description of the assumed cellular dynamics and behaviors would help. These results will interest microbial ecologists, who study overflow metabolism in the context of cellular growth and division, often in glucose-limited environments. Does glucose import in this system lead to growth and division? Do the opposing strategies to optimize rate or yield of ATP (Fig. 2) correspond to r/K strategies (in yeast, these descriptions correspond exactly)? If the cells are not dividing, what do these strategies describe?*

In the revised manuscript, we have included some more detail on the assumptions behind the simple metabolic reaction model and intercellular coupling via lactate diffusion. In essence the simple metabolic network we considered for single cells does not include a biomass term since it is meant to encode only for energy production during the adaptation phase. As we wrote in response to Reviewer 1's point 1.4C, at odds with previous work on maximum entropy modeling metabolic networks focusing on microbes, the function $h$ does not present a growth rate term. To summarise, cells do not undergo a significant amount of cell division or growth over the time interval of our experiments, and this was therefore not incorporated into the model. In this sense, as outlined in the corresponding response and stated again here for the sake of this reviewer's particular interest in application to microbial metabolism, the model more realistically applies to the adaptation (lag) phase with generation of ATP purely for maintenance requirements. It would, however, be possible to conceive an extension of the same framework beyond the scope of the study that includes both cross-feeding and growth into an appropriate model for microbial communities. We hope to address such a model in future studies and again thank the reviewer for highlighting their interest in this direction of our work.

2.2 *Furthermore, is glucose limiting in this system, and is it depleted over the time course of the experiments described? (Why isn't a glucose concentration time course shown, similar to those shown for lactate and oxygen?) The description (page 9, top of first column) of adaptation to the medium, where average glucose and oxygen import decrease over time, brings to mind a microbial culture reaching stationary phase after glucose has been depleted. Yet the authors describe this process as movement "toward the state of optimal ATP yield in the balanced regime." Are there any commonalities between these processes?*

Glucose uptakes and concentrations can be inferred from lactate and oxygen fluxes, and we can safely assume that any change in glucose concentration is negligible in limiting the uptake. The initial glucose concentration in the medium (DMEM) is 25 mM, leading to an estimate for the diffusion limitation on uptakes of $U_{max,glc,diff}/K \simeq 170$ mmol/gh, which is much larger than the limit uptake we estimate from the maximum number of glucose transporters ($\simeq$ 1mmol/gh). Over the six hours of the experiment, we estimate that glucose depletes at most by 10%. Such an amount does not significantly limit the uptake rate. We have now added a new section to the Supporting Information (S8), devoted to explaining the issue of glucose limitation in detail.

We furthermore agree with the reviewer that many aspects of our results are reminiscent of the situation encountered in microbial growth, especially in diauxie, when certain bacteria make the conversion from (e.g.) glucose to ethanol/acetate. This is indeed occasionally viewed as the analog of the Warburg effect in microbial cultures. However, we emphasize that: 1) as stated above, we are in a regime when both the effects of growth and glucose depletion are negligible; and 2) our predictions for when overflow metabolism occurs are based on a quasi-steady state model and correspond instead to the breakdown of coordination of intercellular lactate exchange, rather than a change of extracellular substrate concentrations per se. However, in the revised manuscript we have now extended the model to include situations when a certain level of exogenous lactate is available to the culture (see the new Section S7 in the Supporting Information). As shown in Figure 4 of the main text, we describe how levels of exogenous lactate, such as that produced by cells at previous time points, can reshape the critical behavior. In this sense, this extension of the model encompasses a phenomenon somewhat analogous to the dynamical regime the reviewer describes– where activity of the culture updates the environment, which in turn directs re-adaptation of the culture.

2.3 *More generally, can this model be applied to different systems to understand overflow metabolism? While oxygen remains high in this system, what would the model explain about hypoxic tumor microenvironments or anaerobic fermentation? The phase diagram (Fig. 3B) suggests that transition to the overflow state is triggered more easily by glucose import in low-oxygen environments. Is this true, and if so, what is the relevance to high-lactate tumors and high-ethanol fermentation in which yeast overflow products lead their own populations to collapse?'*

The referee made an important remark since constraint based models have been particularly successful in modeling microbial growth and in particular overflow metabolism. Constraint based modeling has been used to analyze bulk data under the simplifying assumption of an objective function maximization, and our maximum entropy framework could in principle accommodate for the analysis of single cell data on microbial growth. This has been shown to be the case for growth rate distributions in previous works, and the framework we are suggesting here could eventually be applied to the study of microbial overflow within a single-cell perspective. In this respect we would need to extend it both from an experimental and modeling viewpoint. From an experimental point of view, the current framework has been developed having in mind the particular data and system at hand, that is time-resolved single cell fermentation fluxes from a tumor-stroma co-culture during the adaptation phase obtained by monitoring the microcellular environment via fluorescent nanofibers. In thinking about applying such a monitoring system to microbial cultures it is however important to remark that the fibers have a size that is comparable to the one of the cells themselves ($0.3 - 5\mu$m) and this could hamper the possibility of achieving a spatial resolution able to resolve for exchanges.

To the best of our knowledge, a successful method to probe for single-cell metabolic activity for the smaller spatial resolution of microbes consists in performing mass spectrometry at the nanoscale level (Critical Reviews in Biotechnology 36.5 (2016): 884-890.). These methods are known to resolve for single cells turnover times for the main elements (e.g.

carbon and nitrogen: Environmental microbiology 17.7 (2015): 2542-2556), and in principle the extension of constraint-based modeling to sub-optimal cases and including intercellular exchanges could help to analyze these data toward the possibility of performing single cell metabolic flux analysis. From a theoretical perspective, it would be important to include biomass generation into the model, up to to the possibility of considering truly constraint-based metabolic network models for each single cell, to which we would add the intercellular diffusion constraints we considered here. This would lead to a heavier computational problem for which solutions could be envisaged, e.g. the use of approximate methods for sampling (Nature communications 8.1 (2017): 14915). An unavoidable caveat of nanoscale mass-spectrometry data would be its destructive character, which would lead to just one timepoint.

Concerning the oxygen constraints, we have included in the revised Supporting Information a new section showing more in detail why they are effectively immaterial for our particular experimental conditions (Sec. S8), as well as simulation results showing how they would start to matter for oxygen concentrations roughly half of the current values (or equivalently doubling cell density). We plan in the near future to perform data analysis within this modeling framework for very recent datasets coming from oxygen nanofibers experiments [Grasso et al. "Highly sensitive ratiometric fluorescent fiber matrices for oxygen sensing with micrometer spatial resolution." Bio-Design and Manufacturing 7.3 (2024): 292-306] and their experimental extension to cases of interest.

Finally, about analyzing culture collapse in highly polluted environments: this is a phase that has been understudied in constraint-based metabolic modeling, as its main focus is on the completely opposite regime of exponential growth. We agree with the referee that studying the death phase would be crucial to fully understand overflow phenomena. A study of the theoretical basis of cell death within contemporary systems biology modeling schemes, in particular metabolic modeling, has been recently performed by Dr. Y.Himeoka and collaborators (Himeoka, Y.et al. (2024). Theoretical basis for cell deaths. Physical Review Research, 6(4), 043217; eLife 13 (2024); Physical Review Research 4 (4), 043223 (2022)).

2.4 *The model is described as two-dimensional. A justification for this assumption is missing, or a discussion of how results might change in three dimensions.*

We thank the referee for raising this issue that has been already considered in the reply to reviewer 1 (1.4A and 1.6). We chose to model primarily a two-dimensional arrangement of cells, since this replicates the types of cultures for which we have experimental data, where cells adhere to their substrate. However, we are keen to highlight that we can equally well represent a culture in three-dimensions since our flux coupling constraints depend only on absolute distances between cells. To illustrate this, we have now included simulations of the model corresponding to alternative three-dimensional arrangements of cells in the Supporting Material (S9.3). We observe that when cell density increases to levels such as those perhaps expected in cell spheroids, we expect limiting concentrations of substrates and oxygen to require an alternative model beyond the scope of that considered here.

2.5 *"Our analytical expression for" is mentioned on page 6, top of second column, but no expression is referenced.*

We thank the reviewer for pointing out that this way of expressing how we obtained $\langle u_L \rangle$ when opening the corresponding paragraph may not be clear. We have now directed the reader to SI section S7 where the self-consistent equations are stated and replaced the opening line of the next paragraph with: "The numerical solution for $\langle u_L \rangle$ versus $\beta_G$ at fixed $\beta_O$ quantitatively reproduces the sampling results of the previous section...".

2.6 *Why is lag phase, or the population's adaptation to the medium, important to study? And why does the time course begin at 1 hour, and not earlier?*

Studying the lag phase, i.e. the initial period when a population adapts to a new environment before active growth, is particularly important -we believe- in the context of cancer cultures because it provides insights into the cellular and molecular mechanisms that govern cancer cell adaptation and subsequent proliferation. Cancer cells often face diverse microenvironments (e.g., changes in nutrients, oxygen levels, and pH) and during the lag phase, cells adapt by reprogramming metabolic pathways. In particular we do observe medium acidification (Warburg effect) in this phase.

The probing timescale in original experiments was 8 min, that is the time course starts 8 min after seeding. In the original submission we only decided to show snapshots at 1 h intervals to save space and for sakes of clarity of the resulting diagrams.

2.7 *The description of the process of inferring lactate fluxes for Fig. 4 (page 7, first column) starts with previously obtained lactate fluxes, which are then used to infer $\beta_G$ and $\beta_O$. This logic sounds circular.*

We apologize for the confusion. This quite understandably stems from the two separate ways that single-cell lactate fluxes have been calculated: 1) the *observed* single-cell lactate flux values calculated indirectly from pH-sensitive nanofibre experiments (previous publication), and 2) the *predicted* single-cell lactate flux values from the model (current publication), for a given value of $(\beta_O, \beta_G)$. Here, we use maximum likelihood to infer values for $(\beta_O, \beta_G)$ that generate *predicted* single-cell lactate flux that best match their *observed* experimental values. We have now updated the main text and Supplementary Information to clarify any confusion on this issue.

2.8 *Saturated mitochondrial capacity should be defined in terms of the model (page 9, first column). In the current version of the text, it is unclear how the fraction of saturated mitochondrial, shown in Fig. 5, is obtained. I also couldn't find this in the supplement.*

We have used a value of 2 mmol/gh (66% of $U_O$) for mitochondrial saturation. We have added it in the new version of the manuscript, and thank the reviewer for highlighting this omission.

2.9 *In the Discussion, what results do statements about heterogeneity refer to? Should the reader refer to plots of variance in Fig. 3? This feels like an unexplained result.*

We thank the reviewer for the remark. In the revised version of the manuscript we write: "Finally, our results confirm that heterogeneous cell populations can be described effectively by Maximum Entropy distributions like (9) [38, 39, 50–54], suggesting that, at least in some conditions, the constrained maximization of population-level variability might be a reasonable objective for multi-cellular systems." The whole subsection "Inverse modeling experimental data" has been amply revised to show that indeed we can capture the heterogeneous trend of the single cell flux data within the maximum entropy framework, in particular panel 3B, middle (for the standard deviation) and bottom (for the intercellular correlations). Interestingly such trends on the heterogeneity of the observed metabolic fluxes cannot be reproduced in a non-interacting model made of uncoupled single cell (limit of low density) as we explain in the new sections S10 and S11 of the Supporting Information (see in particular Figs S10-12 and S15-S17).

Again, we are very grateful to the Reviewer for multiple useful comments and suggestions.