

Dear Prof. Van Cleve,

we thank you and Drs. Peter Conlin and Matt Herron for your positive assessment, thoughtful comments and constructive criticism.

We realized that some parts of the manuscript were insufficiently clear or lacked precision, and reworked them so as to streamline the presentation and focus our arguments.

We provide here a brief summary of the main changes (marked in blue in the manuscript), and later proceed to answer comments point-by-point.

1. We have rewritten the end of the Introduction, substantial parts of the sections 'Strategies of interacting strains', 'Cell-level strategies' and 'Discussion' in order to make it easier for the reader to follow the logic of our reasoning.

In particular, this involved addressing the scaling-up of games played by cells to effective descriptions at the level of the population. We now conclude the section 'Strategies of interacting strains' with discussing the potential limitations of a strain-level view (even when effective games are considered). This theme – that is indeed central to our argument – connects to the beginning of the 'Cell-level strategies' section, and is touched upon again in the Discussion.

2. We added a table (Table 1) that summarizes the three main conceptual models that come into play when considering cell-level strategies, and their evolutionary implications.

3. We shortened some parts of the manuscript that took the focus away from the main narrative. We have displaced in a Supplementary Information document the paragraphs detailing the experimental evidence of phenotypic effects on spore bias in monoclonal populations. Although we think that it is useful to appreciate the diversity of possible non-genetic determinants of spore bias, their evolutionary implications are similar, and we have thus decided to resume them in the section 'Cellular 'lotteries''. This section refers to the SI, where we also provide a synthetic table of the phenotypic determinants and relative references (Table S1).

4. We removed the 'Conclusions' section, for concision, because of its overlap with the 'Discussion'.

We feel that these changes, prompted by your and the Reviewers' comments, have greatly improved the focus of the manuscript and streamlined the presentation. We are confident that you will have a similar perception.

With best regards,

Silvia De Monte, on behalf of the authors

## Editor

In this manuscript, the authors describe two different conceptual perspectives for understanding the evolution of aggregation and collective behavior in the social amoeba *Dictyostelium discoideum*. The first perspective is the "strain-level" where empirically strain frequencies are used to determine the success of cooperative (less spore bias) or non-cooperative (more spore bias) strategies. The second perspective is the "cell-level" where individual cell fates, spore or stalk, are affected both local biotic and abiotic conditions and by stochastic forces. The paper has a lot of excellent detail about how aggregation and cooperation in *Dictyostelium* functions and might be evolutionarily stable. The "cell-level" perspective highlights a number of important mechanisms that contribute to spore or stalk bias including the cell-cell signaling, cell position, and cell-cycle stage. These mechanisms suggest there is important and understudied complexity in the experimental results of chimeric mixtures and suggest evolutionary models must account for these mechanistic details in order to truly describe how aggregation evolves and is maintained in *Dictyostelium* lineages.

Two reviewers have read the manuscript and agree that preprint is interesting and provides a valuable perspective. They provides a few important areas for improvement that I think the authors should consider.

One area that I would like to highlight specifically is the reviewer's comments about the "the balance of arguments in favor of the strain-level vs. the cell-level perspective". I agree here with the reviewer that manuscript reads a bit more as an exposition on the importance of the cell-level perspective and less of a full comparison of the benefits and drawbacks of both approaches. I also agree that simply signaling this goal earlier in the paper would be a good way to address this issue.

We agree that the manuscript weight is displaced more towards the mechanistic, cell-level perspective. This responds on the one side to the fact that several reviews already focused on the strain-level perspective, to which evolutionary biologists are more familiar. On the other side, we wished to bring attention to facts that are perhaps better known in cell and developmental biology communities, and that are nonetheless important to define the eco-evolutionary dynamics of this organism.

We have nonetheless intervened to bring our presentation to a better balance by:

1. Improving the narrative thread, from the introduction to the discussion, stressing at every step the relevance to evolutionary biology.
2. Moving to the SI the sub-section that chiefly deals with cell and developmental biology, while retaining in the main text only the information that is most relevant in an evolutionary perspective .

**One place that I think the manuscript needs more substantial modification is in its description of the mathematical theory in relationship to the strain vs cell-level perspective. On page 9 in section 3, the authors suggest pure strategy models are sufficient for the strain-level perspective and mixed strategy models are necessary for the cell-level perspective. In actuality, the cell-level perspective doesn't ne-**

cessitate the use of mixed strategies any more than the strain-level does. A pure strategy can be deterministic or probabilistic. In fact, many models of cooperation use a continuous variable to measure the level of cooperation, which conceptually is no different than if that same variable measures a continuous probability of cooperating. Mixed strategies become relevant when one considers the possibility of a mixture of discrete pure strategies. But in many cases this is indistinguishable from a continuous of pure strategies where the strategy is a probability.

The second issue on page 9 is that the paragraph suggests that simply by using a mixed strategy, certain models allow coexistence of different behaviors (stalk vs spore I assume). However, these models really show an equilibrium with both behaviors because they setup a game that is no longer a simple prisoner's dilemma (PD); rather, these games are likely snowdrift (SD) games where a mixed strategy or intermediate value is stable. In other words, its the change of the game structure in these models, not their consideration of mixed strategies per se, that leads to coexistence. This applies to n-players games too where some n-players games result in PD like games and others have nonlinearities that lead to SD like outcomes, but being an n-player game per se doesn't result in nonlinearities (e.g., "Such games naturally introduce frequency-dependent payoffs and nonlinearities" on page 10).

In a few other places the authors suggest that multiplayer games add additional complexity that requires new game theoretic approaches rather than traditional deterministic approaches (the paragraph on page 21 starting "In evolutionary game theory"). This also isn't true (see for example Peña et al 2014 J Theor Biol and Peña and Nöldeke 2015 J Theor Biol).

In general, the authors should take more care about connecting any specific feature of the strain or cell perspective to a technical limitation of a specific game theoretic approach or model; its much more likely that specific models made specific biological assumptions such as regarding the payoff structure of the game the organisms play that resulted in the model's predictions rather than a specific technical aspect of the model analysis.

We thank the editor for his thorough reading of the manuscript! We realize that our discussion on the application of game theory at different levels was unclear and needed to be refined. Thank you, in particular, for pointing to literature we had not previously considered, and that we have now added in the list of references.

We agree that defining different kinds of strategies at the cell or, by means of appropriate averages, at the strain level is not per se problematic from the point of view of evolutionary game theory. Also, that it can be useful to ascertain in models that specific mechanisms - e.g. kin recognition - have the effect of transforming interactions that are assumed to be conflictual at one scale into effective interactions that, at a larger scale, are not.

However, not all mathematical representations share the same degree of biological realism. For instance, let us consider that strains are players that have a mixed or probabilistic strategy. Each time the strains play together (thus, form a chimera), then, their behaviour as cooperators or cheaters would be decided prior to interaction (before aggregation), and this decision may change if they meet again. Since spore bias is highly

reproducible in controlled environmental conditions, we think that it would be difficult to defend this as a relevant model for *Dictyostelium*, though it is mathematically perfectly sound.

We have therefore decided to keep the perspective that strains play pure, non probabilistic strategies (where a pure strategy does not mean that all cells of the strain play a pure cell-level strategy, becoming exclusively spore or stalk). We have however made clear in concluding the section 'Strain-level strategies' that if such pure strategies are the effect of single-cell behaviour, then it is well possible that the game played at the level of the strains does not give rise to an evolutionary conundrum (e.g. it could be a snowdrift game, where polymorphism is to be expected and does not require an ad-hoc evolutionary explanation). As the interest of labelling strains as 'cheaters' largely relies on the assumption that they pose an evolutionary problem, we think that it is worth making these distinctions, though we recognize this was previously rather clumsily done.

We hope our message and the connection with experimental observations at the cell and strain levels is more clear now, after introduction of the following changes:

1. discussion of the biological meaning of assigning strain-level strategies, lines (lines 281-303)
2. improved presentation of cell-level strategies and of the corresponding population-level effective games, lines 322-350
3. additional discussion of the relation between cell-level behaviour and the corresponding population-level null model for evolutionary dynamics, lines 636-651

### Specific comments

**Page 5: "many rounds of the game". In evolutionary game theory, this really is many generations.**

We have now specified on lines 144-147 what corresponds to the 'players' and what is the 'game'. Thank you for stressing that this important point was not clear.

**Page 5: "cost" and "benefit" should be qualified by "fitness" (i.e., fitness cost and fitness benefit).**

We changed the wording when we first talk about costs and benefits, corresponding to Hamilton's qualification. In other parts of the manuscript, we left the shorter notation for simplicity, as we do not think that that influences our arguments.

**Page 6: where citing Fletcher and Doebeli 2009, the authors should also cite Queller 1992 Evolution.**

As pointed out by Reviewer 2, the aim of this paragraph was to stress that genetic relatedness is one of many possible ways of achieving assortment, that is eventually what underpins the advantage that cooperators have on cheaters in a structured population. As this point was clearly not sufficiently elucidated, we have rewritten this paragraph (lines 163-166).

**Page 7: "assemble locally" should be "assemble from locally".**

Changed.

**Page 9: "still contrasted". Not clear what this means.**

The concluding paragraphs of the section have been replaced.

**Page 12: "lead Maeda" should be "led Maeda".**

Changed.

**Page 13: "back of the slug" and "rear form the stalk". What is different between "back" and "rear"?**

We thank the reviewer for pointing out this error. Pre-spores cells are located at the back of the slug whereas pre-stalk cells compose the front region. This sentence has been corrected.

**Page 17: "Fruiting bodies with large heads may be more prone to collapse and would then reduce the potential to disperse of both strains, thus undercutting the reproductive success of the cheater itself." This kind of feature is exactly what sets up the payoffs for a SD game instead of a PD game!**

We agree that several strain-level observations are more consistent with a SD rather than a PD game. However, this is how the game is generally viewed, because in the case of a SD game, the coexistence of cooperators and defectors should not be deemed problematic at all, and actually should be expected. Moreover, since it is unclear how cell-level features turn into a population-level SD, it is hard to know when 'cheating' should be taken as a threat to multicellular organization, and when not. But this is a very important point that we agree should be discussed upfront and not only when speaking of frequency-dependent bias. We now discuss the possibility that the strain-level game is a SD on lines 302-304 and 480-485.

**Page 18: "allows to" should be "allows us to".**

We have removed the corresponding sentence.

**Page 18: "Variation...respectively. I don't think this is an accurate description of the indirect genetic effects approach.**

We changed it to "the influence of the competing strains on the focal strain social behavior".

**Page 19: "statistical description of the outcome of interactions does not inform on the underlying processes.". This seems to reference multilevel selection or kin selection approaches. These approaches are not purely statistical and usually do build on mechanistic biological assumptions.**

We have expanded this paragraph (lines 583-587), and we hope that it is now more clear.

**Page 21: "In evolutionary game theory...processes". I'd ditch this whole paragraph.**

We have rewritten this paragraph in relation with the previously discussed scaling up from individual to population interactions (lines 636-651). We hope that it is now more clear.

**Page 22: "allow nowadays" should be "allow us nowadays"**  
Changed.

## **Reviewer 1**

### **Summary**

**This paper presents an overview of the social conflicts that can arise in *Dictyostelium discoideum* when multicellular structures are formed by the aggregation of genetically heterogeneous cells. In particular, this work is focused on the causes and consequences of spore bias, or the over/under- representation of certain genotypes in the spore mass relative to their initial frequency in a chimeric group. The authors begin with a brief introduction to aggregative multicellular life cycles, the difficulties associated with measuring differential fitness of co-aggregating genotypes, the problem of ‘cheating’ during multicellular development of the fruiting body, and the question of why the existence of cheaters does not doom collective function altogether. The question of why cheats (specifically, genotypes that exhibit positive spore bias) don’t doom collectives is then addressed from two different conceptual perspectives for understanding social conflicts: the strain-level and the cell-level perspective.**

**This article provides a comprehensive review of an interesting topic and I think these authors are providing a valuable perspective on how we conceptualize social interactions in microbes. I especially enjoyed the discussion section, in which the authors lay out the key questions they believe must be addressed in order to build adequate predictive evolutionary models. In fact, I would love for the themes introduced in the discussion to be raised much earlier in the manuscript so that they could be carried throughout the text! This could be helpful because it wasn’t always obvious to me how the experimental data being summarized related back to the central themes of the manuscript.**

We thank the Reviewer for his appreciation of our work. We realized that the presentation needed streamlining, and we intervened in several points to make the message more clear and to enhance the relation between the different parts of the manuscript (see common response to the Editor and Reviewers).

**For example, while it is true that physiological state and cell cycle phase influence developmental fate, it is unclear whether these factors are expected to have differential effects on the developmental decisions of cells of different genotypes or if cells of all genotypes are influenced similarly by these factors. If there is no differential effect of environment, would the incorporation of these sources of phenotypic heterogeneity change our predictions about how the system will evolve? If so, how? For me, it would be extremely helpful to have a table summarizing the major sources of phenotypic heterogeneity, how they influence cell developmental fate, and how model predictions might change when these sources of variation are incorporated. The fact that “different conceptualizations lead to different predictions” is a key takeaway message of the paper being highlighted in the conclusions section.**

We have extensively changed the section 'Cell-level strategies' in order to make a more direct connection between the model chosen for describing cell-level strategies and evolutionary consequences, as summarized in Table 1. We moved the section on different sources of bias induced by phenotypic heterogeneity as Supplementary Information, and provide there a table (Table S1) summarizing different mechanisms that can decouple genotype from reproductive success. We then discuss the evolutionary consequences of such decoupling in the section 'Cellular lotteries'.

**Another area I had some difficulty with was the balance of arguments in favor of the strain level vs the cell-level perspective. The abstract sets up an expectation that this review will be a balanced comparison two competing conceptual frameworks for understanding (and perhaps modeling) social conflicts in *Dictyostelium discoideum*. However, the cell-level perspective receives a disproportionate amount of attention and seems to be the better supported of the two conceptual frameworks based on the results presented. If this is indeed the perspective of the authors, this argument could be made early on in the manuscript and each consecutive subsection in the "Cell-level strategies" section could serve as further support for the need to consider cell-level properties when performing experiments and building models of *Dictyostelium* social evolution. If this is not the perspective of the authors, some further consideration could be given to how strain-level models might be able to accommodate some of the relevant sources of phenotypic heterogeneity (as discussed in the stochastic vs. deterministic models subsection of the discussion).**

We recognize that the weight is not equally distributed in our manuscript between the different approaches. This partly reflects the fact that *Dictyostelium* has been a model system for cell and developmental biology long before it became one for evolutionary biology, and, correspondingly, a larger literature exists on cell fate determination than on evolutionary dynamics. Moreover, evolutionary experiments and theory have already been reviewed elsewhere, and mostly interpreted in a strain-centered perspective. Our goal here was to step back from the assumptions that are involved with representing interactions at the strain level - that in the current version we have tried to evidence - and to examine how taking different points of view leads to different expectations as to the evolutionary questions that this system poses. We hope that the changes we introduced in the Introduction and throughout the manuscript now make our perspective stand out more clearly.

**Minor comments:**

**In the abstract where it says, "cheater populations are selectively advantaged," I might add "when interacting with cooperators".**

We thank the reviewer for this remark and agree that this information was missing. We added it to the revised version of the manuscript on line 15.

**The final paragraph of the introduction (pg 4, bottom) seems to be a concatenation of two different versions of the same paragraph. This starts about halfway through the paragraph with: "Here we take a step back...; A fundamental dis-**

**inction...; We first consider...". One version, or the other, should definitely be deleted.**

We have entirely rewritten the last paragraph on the Introduction.

**In the "Strategies of interacting strains section," I'm wondering if the conditions for a Prisoner's dilemma have been fully demonstrated in *Dictyostelium*. If so, pointing readers to the relevant references would be appreciated. If not, perhaps a short explanation of why this is the expectation?**

To the best of our knowledge, the conditions for a Prisoner's dilemma have not been properly demonstrated in *Dictyostelium*, though the relevance of this game has been claimed based on indirect evidence. The point we wanted to make is that this assumption underscores the fact that cheaters are supposed to constitute an evolutionary threat to multicellular function. If one calls cheating a strategy that does not confer a long-term evolutionary advantage, but just a short-term, transient and possibly variable advantage, then what needs to be explained is not that cooperation persists (it does in the null model, as pointed out by the Editor), but how do single-cell properties translate into population-level behaviour.

We hope that this point is clear in the revised version of the manuscript.

**Also on the topic of the Prisoner's dilemma: the statement that "cheating is always better than cooperating, irrespective of the other player's strategy" is, of course, accurate. However, could it also be made clear that the Prisoner's dilemma also requires the condition that mutual cooperation is more beneficial than mutual defection?**

Your are right, we added this condition on line 143-144.

**Has Simpson's paradox been invoked to explain the success of cooperators in heterogeneous populations of *Dictyostelium*? The assumption of differential productivity of groups with higher a proportion of cooperators seems like it could be reasonable. This could provide an explanation for how cooperation can persist even without mechanisms for positive assortment.**

We are not aware of applications of Simpson's paradox to *Dictyostelium*. However, its application depends again at what level one places the players. If players are cells, then it would predict that cells of a cooperating strain win in an aggregation with a cheater strain, that is not what is observed. If it is applied at the level of strains, then the way strains would be divided into groups of interacting strains is not evident.

Nonetheless, we agree that the Simpson's paradox illustrates perfectly cases when the population-level outcome of a cell-level cooperative behaviour is counterintuitive, therefore we use it as an example on lines 331-339. Moreover, we discuss the effects of grouping, or the mesoscale organization of cells into multicellular aggregates, in relation to their effects on population-level aggregated measure, on lines 193-199.

## **Reviewer 2**

**The preprint contrasts two approaches to modeling social conflicts in the social amoebae, one based on strain-level descriptions and one based on cell-level descriptions. Numerous examples are given of cell-level processes that might, if ig-**

nored, mislead evolutionary predictions based on strain-level models. This is an important distinction, and as such the preprint makes a valuable contribution. In addition, descriptions of aggregation behavior, mechanisms, and genetics are sufficiently thorough that the preprint serves as an adequate review of these aspects of Dictyostelium biology aside from its central argument. Depending on the authors' goals (and possibly on the journal they might submit it to), these descriptions could be substantially shortened without undermining the preprint's central message.

In the Discussion the authors briefly address the conditions under which the cell-level approach is likely to yield different evolutionary predictions than the strain-level approach, and this is one point that I think would be worth expanding on. Doing so could also be left as a future direction, but formally defining the conditions or assumptions under which the two approaches yield equivalent predictions would substantially increase the value of the work.

We are glad the Reviewer found our work interesting! And we agree that our previous version was a bit meandering. Following also the recommendations of the Editor and the other Reviewer, we have tried focus more clearly on the evolutionary implications of different ways of formalizing social behaviour in this system. We moved the details of how phenotypic variation can affect spore bias in the Supplementary Information, and introduced a table summarizing the three main models of cell behavioural choice that we discuss in the cell-level section, together with the associated biological mechanisms and evolutionary implications.

In the Discussion, we stressed that strain-level and cell-level approaches do not need to differ in their evolutionary predictions (at least, as long as one recognizes that 'cheating' and 'cooperation' are not necessarily strategies associated to evolutionary conflicting outcomes). Their main differences is in the integration of cell-level observations and in the transparency of the relationship between different levels of description, which is essential if one wants to make predictive statements. Hopefully the conclusion of the section on 'Strategies of interacting strains' makes this clear now.

#### **Minor comments:**

**Introduction, paragraph 1: "defended by predators" should probably be "defended against predators".**

Changed.

**Introduction, paragraph 3: the abbreviation cAMP has not previously been used and should probably be spelled out here.**

We added the spelling of this abbreviation when we first mention it in the manuscript.

**Introduction, paragraph 4: "Giving up one's own descent" is an unusual wording, and I'm not sure it conveys the authors' intent. "Giving up one's own descendants" might be preferable.**

Changed.

**Introduction, paragraph 6: the cheating strategy in game theory is usually re-**

**ferred to as "defect" rather than "defective".**

Changed.

**Section 2, paragraph 3: it may be worth noting that the Fletcher Doebeli model breaks with the foregoing description of kin selection in that cooperators need not share an allele at a particular locus, i.e. it does not require identity by descent.**

Thank you for this suggestion: we realized that the important message in this sentence was left implicit. This also caused a misunderstanding with the Editor. We have modified this sentence so as to stress the point you raised (lines 163-168).

**Last paragraph in the 'genetic assortment' section: social amoebae biologists probably understand the significance of washing whole dishes before spore count, but I don't.**

"Washing whole dishes" means collecting every spore produced at the end of the aggregation. This is done by flooding the Petri dish over which fruiting bodies developed with buffer, and then collect the liquid. Explanation of the procedure is however no longer relevant, as we have rewritten that part of the manuscript in order to make it more concise.

**4th paragraph of the Cell phenotype through development section: "cells at the back of the slug tend to turn into spores, whereas most of those at the rear form the stalk." I suspect one of these should refer to the front.**

Corrected.