Analysis of an experimental transition in individuality challenges the need to assign traits to levels

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Abstract

A major evolutionary transition in individuality, at any level of the biological hierarchy, occurs when groups become capable of participating in Darwinian processes as units of selection. Identifying transitions in individuality can be problematic because apparent selection at one level of the biological hierarchy may be a by-product of selection occurring at another level. Here we discuss various approaches to this 'levels-of-selection' problem, drawing attention to the futility of designating traits to hierarchical levels prior to determining the level of selection. While individuals can exhibit traits that are maintained by selection at any level in the biological hierarchy, terms synonymous with 'collective (group) trait' or 'particle trait' are often applied prematurely. We argue that traits should not be assigned *a priori* to a hierarchical level, but may respond to selection at different levels depending on environmental conditions. This point is illustrated by an experiment in which groups of the bacterium *Pseudomonas fluorescens* reproduced via life cycles involving soma- and germline-like phases. The rate of transition between the two cell types was a focus of selection, and might be regarded as a property of groups, cells, or even genes. Philosophical analyses reveal that this trait evolved to be higher in one treatment as a result of its emergent relation to group fitness (contributing to the evolution of individuality), and lower in another treatment because of cell-level selection. An *a priori* designation of transition rate as either a 'group trait' or a 'cell trait' may have masked the true level of selection.

Introduction

Evolutionary transitions in individuality are central to the emergence of biological complexity. During the transition from single cells to multicellular organisms, groups of cells – on multiple occasions (Baldauf 2003) – have emerged as 'Darwinian individuals' capable of evolving by natural selection – as units of selection in their own right. The causes of these transitions are unclear. Nonetheless, philosophical and theoretical literature has provided insight into the fundamental requirements for evolutionary transitions to take place (Buss 1987; Maynard Smith and Szathmáry 1995; Michod 1999; Bonner 2000; Godfrey-Smith 2009; Mikhailov et al. 2009; Libby and Rainey 2013a; Shelton and Michod 2014; Black et al. 2020). Recently, the question has also received interest from the field of experimental evolution (*e.g.* Ratcliff et al. 2012, 2013; Koschwanez et al. 2013; Hammerschmidt et al. 2014; Herron et al. 2019; Rose et al. 2020), which focuses more on the environmental and ecological circumstances that can establish conditions promoting transitions to multicellularity. Here we attempt to bridge the gap between the fields of philosophy of biology and experimental biology by application of philosophical frameworks to experimental data. In so doing we highlight the importance of careful consideration of the level(s) of selection prior to drawing conclusions based on intuition.

Levels of Selection

The view that the evolution of multicellularity necessitates a transition in individuality is based on a 'multilevel' perspective (Heisler and Damuth 1987; Damuth and Heisler 1988), which maintains that life is hierarchically organised, with 'higher' level units comprised of groups of particles from the level below. For example, eusocial colonies are made up of multicellular organisms, which in turn are integrated groups of cells. The very existence of a biological hierarchy is evidence of transitions from groups to Darwinian individuals. It

follows that during the evolutionary history of the hierarchy, groups emerged as units that evolve by natural selection – that is, entities that can acquire adaptations.

The essence of a transition in individuality lies in the capacity for higher-level reproduction, a prerequisite for Darwinian evolution. While group reproduction is necessary, it is not sufficient to ensure that natural selection operates at the level of the group. The archetypal 'recipe' for a trait to evolve by natural selection at a particular level of the biological hierarchy is that the trait under selection must be heritable, and it must covary with fitness (reproductive success) *at that level* (Lewontin 1970). However, covariance is a statistical concept and not a causal one. There *may* be a causal (selective) influence of a given trait on the fitness of a group if the trait covaries with group fitness, but any such covariance may also be misleading (Okasha 2006). Therefore, considering the possibility of natural selection independently at each level is inadequate because an apparent Darwinian process at one level can be a side effect of selection operating at another level – a 'cross-level by-product' (Sober and Lewontin 1982; Okasha 2006; Shelton and Michod 2014). Okasha (2006, p.78-79) frames the 'levels-of-selection problem' as follows:

> *"When is a character[1]-fitness covariance indicative of direct selection at the level in question, and when is it a by-product of direct selection at another hierarchical level?"*

Attempting to understand the evolutionary origin of the biological hierarchy naturally leads to a multilevel view of evolution by natural selection, however alternative reductionist perspectives are also recognised. The supervenience argument, for example, maintains that genuine group-level selection is impossible because all properties of groups depend on those of their constituent

[1] A semantic note: The term 'trait' is used throughout the paper, however 'character' is on occasion used interchangeably with 'trait' when adopting the language of key reference material. 'Group' and 'collective' are also used interchangeably in this manner, as is the term 'particle', which is used by some references to refer to the units that comprise groups.

particles. The traits of the underlying particles ultimately determine both the fitness and character of groups; the whole 'supervenes' on its parts (summarised in Okasha 2012). The argument then follows that there can never be a causal link between a group's character and its fitness because *all* causality is at the particle level. The supervenience argument against group-level selection renders the levels-of-selection problem irrelevant because it makes cross-level by-products ubiquitous.

Perspectives that reduce all evolution to lower levels are not practical frameworks for investigating evolutionary transitions because of limited explanatory power. For example, the supervenience argument is unable to account for how the biological hierarchy itself came into existence and must logically reject the notion of a hierarchical structure in biological systems. Even the reductionist perspectives of Dawkins (1976, 1982) and others, which maintain that genic selection can account for all evolution, are not congruent with the supervenience argument because genes themselves are groups of lower level molecules whose evolution requires explanation. Okasha (2006) reasons that reductionist arguments only show that a trait-fitness covariance at the higher level must be a side effect of *"some lower level causal processes or other,* but not necessarily lower level *selection*". Certainly everything in existence can be explained by its constituent particles, however reducibility to lower-level *selection* is what is important for understanding evolutionary origins. The underlying particle traits on which group fitness supervenes do not necessarily include particle *fitness*, so it does not follow that group fitness always has a lower-level *selective* explanation. Therefore, Okasha suggests that the concept of a cross-level by-product should be restricted to a trait-fitness covariance at one level that results from *selection* operating at another level.

In our view, if the level of *selection* is what matters, then characters or traits themselves need not be assigned to any particular level. A trait may appear intuitively to belong to a particular level, but other cases are less clear. For example, the trait 'height' is necessarily the height of some entity that exists at a particular level, e.g. the height of a tree, or a termite mound. On the other hand,

a bistable switch between two cell phenotypes might be viewed as a property of cells, genes, or even a group of cells (manifesting as the proportion of the different cell phenotypes in the population). Even seemingly clear-cut cases may be less obvious when informed by the level of selection rather than by intuition. The height of a termite colony, for example, may not play a role in selection between colonies, but could be a by-product of selection acting on individual termites within a colony for some trait that can only be empirically measured as the height of the mound. The distinction between group trait and particle trait is unnecessary and can lead to debates that detract from the real issue of the selective cause of the trait in question. It makes no sense (and in fact invokes circular logic) to utilize the terms 'group trait' or 'particle trait' prior to determining the level(s) at which the trait was selected. If a given trait is not assigned to a particular hierarchical level, discussions over whether it is reducible to particle traits are irrelevant. The relevant factor in determining the level of selection is the level at which a given *undesignated* trait is causally associated with fitness.

The levels-of-selection problem is related to G.C. Williams' (1966) distinction between 'group adaptation' and 'fortuitous group benefit'. In his well-known thought experiment, Williams contrasts herds of deer that run at different speeds. Herds made up of faster deer grow and 'reproduce' (split into new herds) more quickly because slower deer are more susceptible to predation. Consequently, selection for fast deer within herds leads to increasingly faster herds of deer – a fortuitous group benefit. The fitness of the herds (the rate at which they reproduce) covaries with the average running speed of the herd, however this covariance is a cross-level by-product of within-herd selection for faster deer - not a group adaptation, or the outcome of herd-level selection. To assign deer speed as an adaptation of the herd would be to conclude that faster deer were selected *because* of the effect of this adaptation on the reproduction rate of herds. While herds indeed benefit from being comprised of faster deer, causality cannot be attributed to between-herd selection. A fast individual deer does not benefit the other deer within its herd, rather faster deer were selected

because of within-herd competition to evade predation; causality is at the lower level.

The cross-level by-product is conveniently detected in the hypothetical example above because Williams provides two factual statements that are necessary to conclude that the evolution of fast deer is due solely to selection between deer: 1) faster individual deer are better able to evade predators, and 2) herd reproduction occurs as a result of herds reaching a critical size (given by the number of deer within a herd). If these were the only two biological facts then it is straightforward to conclude that the relationship between herd speed and herd reproduction is caused 'from below' by deer-level selection. However, if these two facts were not given, the true selective cause of the evolution of fast deer would be unknowable. Moreover, were *more* biological information provided, the conclusion reached may be altered. Let us imagine that access to more data revealed a trade-off between deer speed and deer reproduction, such that while fast deer are more likely to survive in the presence of predators, slow deer produce more offspring. In this scenario, deer may evolve to be faster or slower depending on the density of predators in the environment. Let us make the assumption that in a low predator density environment, there is nevertheless selection for fast deer. In this case it would be dubious to conclude that increased deer speed resulted solely from within-herd selection. Let us further imagine that herd size is not the sole determinant of herd reproduction, rather new 'offspring' herds are founded after an act of aggression, and such an act is more likely to occur in herds with a higher proportion of fast deer. It is now plausible that these fast herds are maintained, at least in part, by between-herd selection.

The levels-of-selection problem is not often addressed adequately (if at all) in experimental evolution literature. For example Ratcliff et al. (2012) evolved 'multicellular organisms' from the unicellular yeast *S. cerevisiae* by selection for rapid settling cellular clumps in liquid media. The result was evolution of a clustering 'snowflake' phenotype. These groups of cells reproduce via multicellular propagules that separate from the parent snowflake by apoptosis

(cell death) of a single cell at the break point. The selective regime favoured larger snowflakes and higher rates of apoptosis – resulting in higher rates of group reproduction. If snowflakes vary in traits that affect their fitness, and if this variation is heritable, then it is possible that these traits evolved in response to between-snowflake selection. However, to claim that group-level selection – and a transition in individuality - has occurred, it is necessary to show that the observed adaptations not only improve the fitness of the snowflake, but that this improvement is not a by-product of selection acting at the cell-level – a fortuitous group benefit. In this experiment, cell fitness also increased and was correlated with rate of apoptosis. By forming snowflakes that produce a higher proportion of altruistic cell types, recipient cells can grow faster than their non-cooperative ancestors. While it is possible that group-level selection may have contributed to the increased rate of apoptosis, it is also possible that apoptosis increased due to cell-level selection. Despite positive correlations between apoptosis rate and both cell and snowflake reproduction, Ratcliff et al. assigned the apoptosis trait to the snowflake level.

It is possible to identify an evolutionary transition in individuality by determining the level(s) of selection. Groups evolve as higher-level Darwinian individuals when their evolutionary success can no longer be fully accounted for by the evolutionary success (*i.e.,* fitness) of their constituent particles. In other words, a primordial multicellular group constitutes a unit of selection once group functionality, rather than size, becomes the main determinant of fitness (Okasha 2006). As illustrated by the examples above, detecting the level of selection can be problematic because there exists a 'grey area' of mid-transition groups (Godfrey-Smith 2009). In such groups it is unclear whether certain properties are evolving due to selection at the group level, the cell level, or both. We cannot simply assign an evolving trait to a particular level of selection based on assumed benefits to this level. Furthermore, the emergence and evolution of Darwinian properties themselves (*i.e.,* reproduction and heritable variation) at the higher level requires a selective explanation. It is not sufficient to simply transpose the existence of Darwinian properties, such as

variation, from the lower level to the higher (Bourrat 2015); the possibility of selection acting upon this variation is dependent on the mode of group reproduction (Libby and Rainey 2013b; De Monte and Rainey 2014; Hammerschmidt et al. 2014; Rose et al. 2020).

Hammerschmidt et al. (2014) reported the results of a selection experiment involving lineages of the bacterium *P. fluorescens* that differed in the mode of group reproduction. Groups reproduced via a single-celled bottleneck in two selection regimes, however in one regime this bottleneck was an alternative cell type (a 'germ' cell). Crucially, while all groups exhibited the hallmarks of natural selection (reproduction, variation and heredity), improvements in group fitness became 'decoupled' from the fitness of constituent cells only for groups that reproduced via a germline.

This experiment illustrates some difficulties with a simplistic interpretation of the process of natural selection at multiple levels. Recognition of a transition in individuality calls for careful consideration of the possibility of cross-level by-products. Various methods have been proposed for determining the hierarchical level(s) at which the association between a trait and fitness is causal, rather than merely statistical. Here we describe several approaches to tackling the levels-of-selection problem and apply them to the *P. fluorescens* populations. We demonstrate that a key trait evolved in the experiment due to selection at different levels depending on the environment.

The Experiment

The Hammerschmidt et al. (2014) experiment tracked the evolution of groups of the bacterium *P. fluorescens,* which had previously been shown to evolve between mat-forming (soma-like) cells and dispersing (germline-like) states via negative frequency-dependent selection (Rainey and Rainey 2003). When cultured in a static environment, rare cooperative "wrinkly spreader" (WS) mutants invade populations of planktonic "smooth" (SM) cells by forming a

261 surface-colonizing soma-like mat that allows cells to access oxygen. Within the
262 mat, SM "cheating" germline-like cells rise in frequency when rare due to
263 advantages that stem from not producing costly cell-cell adhesive.

264 The frequency-dependent interactions between SM and WS cell types were
265 utilized to implement a multicellular life cycle in which the WS mats were
266 analogous to soma, and SM cells functioned as a germline. This facilitated
267 differential group death and birth, which in turn allowed selection to operate
268 between groups (Fig 1a). After ten life cycle generations, group fitness (relative
269 number of group offspring produced) increased while cell fitness decreased; the
270 fitness of the higher and lower levels became decoupled – a signature of a
271 transition in individuality. Enhanced fitness of evolved groups was attributable
272 to a property selected at the group-level, namely, the capacity to transition
273 through the germ and soma phases of the life cycle (termed 'transition rate'),
274 and was not explained by improvements in individual cell proliferation - the
275 fitness of the SM 'propagules' and the total carrying capacity were both
276 reduced.

277 In parallel to this "cheat-embracing" selective regime, a "cheat-purging"
278 experiment was conducted, in which groups reproduced via a single WS
279 propagule cell (Fig 1b). While group fitness increased in both regimes, in the
280 cheat-purging regime this was correlated with increases in all indicators of cell
281 fitness. The following sections contrast the results of the cheat-embracing and
282 cheat-purging ecologies in light of various perspectives arising from the levels-
283 of-selection debate.

284

285 Emergence

286 One approach to the levels-of-selection problem is to distinguish between an
287 *emergent* trait – a property of a group that cannot be qualitatively or
288 quantitatively reduced to the sum of its parts (Corning 2002), and *non-emergent*
289 (or 'aggregate') traits. How such a distinction is made is not always

290 straightforward because all properties of a group can ultimately be explained by
291 the underlying particles (discussed above). Nevertheless, the 'emergent
292 character requirement' (Vrba and Eldredge 1984; Vrba 1989) posits that genuine
293 group-level selection, that is not reducible to selection at the particle level, can
294 only operate on emergent traits. Okasha (2006) objects to this rule because an
295 emergent trait may be a *result* of selection operating at the group-level, rather
296 than a pre-condition of it. The capacity for group reproduction, which is
297 necessary for group-level selection, is itself a derived, and arguably emergent,
298 trait (Griesemer 2000; Rainey 2007; Rainey and Kerr 2010). It is also possible
299 for an emergent trait to increase in frequency in the absence of group-level
300 selection if it is associated with traits that increase particle fitness, *i.e.*, an
301 emergent trait may inadvertently result from particle-level selection. It is
302 therefore generally accepted that the emergent/aggregate distinction is not
303 useful for solving the levels-of-selection problem (Damuth and Heisler 1988;
304 Okasha 2006, 2012; Godfrey-Smith 2009).

305 Stephen Jay Gould argued that while a given trait need not be emergent to
306 evolve at the level of the group, emergent *fitness* is what matters for group-level
307 selection (Lloyd and Gould 1993; Gould 2002). It is true that groups must
308 acquire the capacity for reproduction to allow the possibility of group-level
309 selection and a measure of group fitness. However, this potential for group-level
310 selection does not rule out cross-level by-products. Group fitness emerged
311 (increased) in both the cheat-purging and cheat-embracing selective regimes in
312 Hammerschmidt et al. (2014) however in the cheat-purging regime this was
313 likely due to a cross-level by-product because the emergent group fitness was
314 correlated with increased cell fitness.

315 Heisler and Damuth (1987) suggest that emergence of the *relation* between a
316 trait and group-level fitness is of fundamental importance for group-level
317 selection. A trait-fitness relation at a higher level is emergent if it cannot be
318 accounted for by a trait-fitness relation at a lower level. Okasha's (2006)
319 formulation of the levels-of-selection problem essentially recapitulates the

emergent relation requirement. It is possible to identify an emergent relation when there is no positive association between particle and group fitness (Fig 2).

A relationship exists between cell fitness and group fitness in the *P. fluorescens* groups that evolved under the cheat-purging regime (Hammerschmidt et al. 2014). This (probably causal) link presents no difficulty in determining the level of selection, however, because the trait in question (transition rate) correlates only with parameters of cell fitness, not group fitness (Fig 3a). It is clear therefore, that the transition rate significantly decreased because of selection acting at the cell level, and the increased group fitness can be attributed to the correlation between group and cell fitness parameters – group fitness supervenes on particle fitness. This is in contrast to the cheat-embracing regime, in which no correlation exists between cell and group fitness in the evolved populations thus the positive relationship between the increased transition rate and group fitness is causal - an emergent relation (Fig 3b).

In Williams' (1966) example, however, there is a positive association between deer fitness and herd fitness, so it is impossible to determine whether the emergent relation between speed and herd fitness is causal from correlations alone – there would be a red line between all three points in Figs 2 and 3 (the same is true for the yeast snowflake example).

Contextual Analysis

Heisler and Damuth (1987) devised a method for detecting emergent relations by analysing trait-fitness covariance from phenotypic data. Contextual Analysis is a generalisation of the selection analysis developed by Lande and Arnold (1983). This approach treats the levels-of-selection problem as a special case of selection on correlated traits, where the dependence of fitness on correlated traits is captured with a standard linear regression model. The covariance of group fitness (Y) with a (so-called) 'group' trait (Z) could be non-zero (*i.e.*, $Cov(Y,Z) \neq 0$) in the absence of a causal link between Z and Y if Z correlates

with another trait that *does* affect fitness. There is a cross-level by-product if *Y* is causally determined by the average fitness of its constituent particles, *W*. The trait-group fitness covariance can be partitioned into two components: direct group-level selection on a trait, $\beta_1 Var(Z)$, and the by-product of particle-level selection, $\beta_2 Cov(W,Z)$:

$$Cov(Y,Z) = \beta_1 Var(Z) + \beta_2 Cov(W,Z) \quad (1)$$

where β_1 is a measure of the direct effect of *Z* on *Y*, controlling for *W*, and β_2 measures the effect of *W* on *Y*, controlling for *Z*.

No relationship exists between transition rate and cell fitness in the cheat-embracing *P. fluorescens* populations (Hammerschmidt et al. 2014). Contextual analysis is therefore not necessary to partition the change in transition rate due to cell- and group-level selection, because $Cov(W,Z) = 0$. The strength of group-level selection for increased transition rate is equal to the covariance between group fitness and transition rate:

$$Cov(Y,Z) = \beta_1 Var(Z) + 0 \quad (2)$$

$$Cov(Y,Z) = \beta_1 Var(Z) \quad (3)$$

As no relationship exists between transition rate and group fitness in the evolved populations from the cheat-purging regime (Hammerschmidt et al. 2014), there is no need to discriminate between levels of selection – decreased transition rate was selected entirely at the lower level:

$$Cov(Y,Z) = 0 \quad (4)$$

These results corroborate the idea that a trait need not be assigned to a particular hierarchical level. The transition rate between the two cell types might be regarded as a property of groups, cells, or even genes, often depending on the inclinations of the investigator. Our view is that transition rate should not be associated with one level because the direction of its evolution can change due to selection operating at multiple levels in differing ecological contexts

(Rose et al. 2020). This trait evolved to be higher in the cheat-embracing selective regime as a result of its influence on group fitness, and lower in the cheat-purging regime because of selection at the cell-level. Does it then follow that transition rate is a group trait in one ecological context and a cell trait in another? To reiterate, the level of *selection* is the focus during an evolutionary transition in individuality. An *a priori* designation of transition rate as either a 'group trait' or a 'cell trait' may have masked the true level of selection, or worse, led to false conclusions.

Additivity

Lloyd (1989) contributed the 'additivity criterion' to the levels-of-selection debate - a notion that was first put forward by Wimsatt (1980). The additivity criterion posits that true group-level selection can only occur when the variance in group fitness does not relate additively (linearly) to particle composition. When there is a linear relationship between group fitness and particle composition, then differences in group fitness can be fully explained by selection at the particle level. A shift in the level of selection occurs when there is a departure from additivity, *i.e*,. there is a non-linear relationship between the particle composition of groups and fitness. The additivity criterion for group-level selection is similar to the criterion of emergent relations: a 'new' non-linear relation between a trait and fitness emerges at the level of the group. However the additional specification that this relation be non-linear adds a crucial layer of information that the emergent relation requirement alone lacks. To illustrate, the emergent relations requirement could not assign selective causality to the evolution of fast deer in the Williams example (discussed above), however the stipulation by Williams that herds reproduce as a function of herd size results in an additive relationship between herd fitness and their composition, rendering the selection of fast deer to be at the lower (deer) level.

404 The cheat-embracing selective regime in the *P. fluorescens* populations clearly
405 results in a non-linear relationship between group fitness and particle
406 composition. SM 'germline' cell types are required at a high enough frequency
407 to be consistently detected during the regime, however a WS mat must also be
408 present to avoid extinction. Therefore, group fitness will be highest at
409 intermediate frequencies of SM cells, and zero if the proportion of SM cells is
410 either 0% or 100%. This non-additive relationship between cell composition
411 and group fitness is not pronounced in the experimental data (published in
412 Hammerschmidt et al. (2014) but re-analysed here) because the wildtype
413 transition rate is so low that the hypothetical 'high SM/low group fitness' groups
414 do not exist in the ancestral populations, and were not selected during the
415 experiment due to their low group fitness (Fig 4a). Nevertheless, a comparison
416 of the fit of linear and quadratic models to the data confirms the non-additive
417 relationship between the proportion of SM cells within a group and fitness.
418 Akaike's Information Criterion (Akaike 1973), which compares the quality of
419 statistical models by giving a relative estimate of the information lost by
420 overfitting a given model, indicated that the quadratic model was 99.99% and
421 99.88% more likely to explain the data than the linear model in the ancestral
422 and evolved groups respectively (Supplementary Table). This non-additive, and
423 therefore emergent, relationship between the proportion of SM cells (determined
424 by the transition rate trait) and group fitness is indicative of selection operating
425 at the level of the group.

426 In contrast, there is an additive relationship between the composition of groups
427 (as determined by transition rate) and their fitnesses in the cheat-purging
428 selective regime. Compared to the quadratic model, the linear model has a
429 70.54% and 69.94% probability of correctly fitting the ancestral and evolved
430 data respectively. The two ancestral lines that had both low group fitness and
431 low proportion of SM cells did not exhibit visible mats because of the invasion
432 of a third cell type, the "fuzzy spreader" (described in Ferguson et al. 2013).
433 These groups were therefore extinguished after one generation and the
434 proportion of SM alone became a reliable predictor of group fitness. In contrast

to the non-linear relationship between the fitness of a group and its composition in the cheat-embracing regime, the additive dependence of a group's fitness on its composition reveals lower-level causality in the cheat-purging regime. Non-additivity in the cheat-embracing life cycle led to a transition in individuality that was not possible for groups in the cheat-purging regime, whose fitnesses depended entirely on particle composition.

Conclusion

The results from the experiments presented in Hammerschmidt et al. (2014) and scrutinized here in further detail demonstrate that a major evolutionary transition to multicellularity is possible when groups of cells reproduce via a two-phase life cycle involving a germline-like cell. Conflict resolution evolved in groups that reproduced without a germline (in the form of reduced transition rate to SM 'germline-like' cell types), however these groups did not evolve individuality. In contrast, during the course of the evolution experiment, a causal relation between the transition rate trait and group fitness emerged in groups that reproduced via a germline-like cell. This emergent relation, in addition to a non-additive relation between the composition of the groups and group fitness, demonstrates that the levels-of-selection problem was resolved, shifting selection to the higher level and revealing new biological individuals free to participate in an open ended manner in Darwinian evolution. Importantly, while the transition rate trait evolved to be lower in one experimental regime due to selection acting at the cell-level, the same trait increased due to group-level selection in the other experimental setup, challenging the common practice of assigning traits to levels prior to rigorous levels-of-selection analyses.

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Declarations

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Conflicts of interest/Competing interests

The authors declare no competing financial interests.

Availability of data and material

Request for materials should be addressed to the corresponding author.

Code Availability

Not applicable

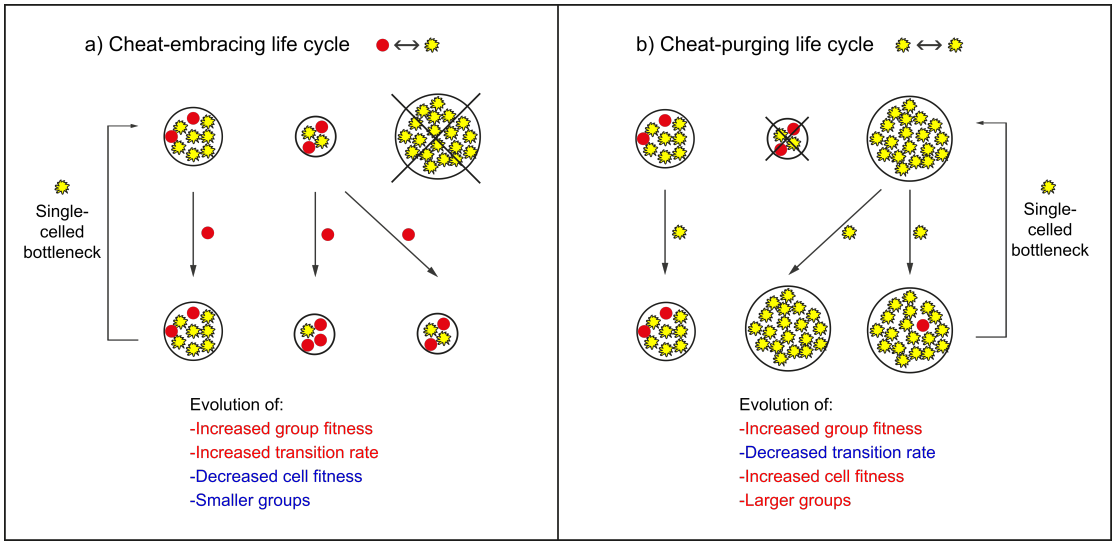


Fig 1. Simplified schematic contrasting the two experimental manipulations carried out by Hammerschmidt et al. (2014). a) In the "cheat-embracing" regime, groups of bacteria reproduced via a germline-like cell: a phenotypically different, non-cooperative cell type. b) In the "cheat-purging" regime, groups of bacteria reproduced via a single-celled bottleneck of the same cell type. Cell fitness is the total cell density, while group fitness is the relative ability of groups to beget offspring groups.

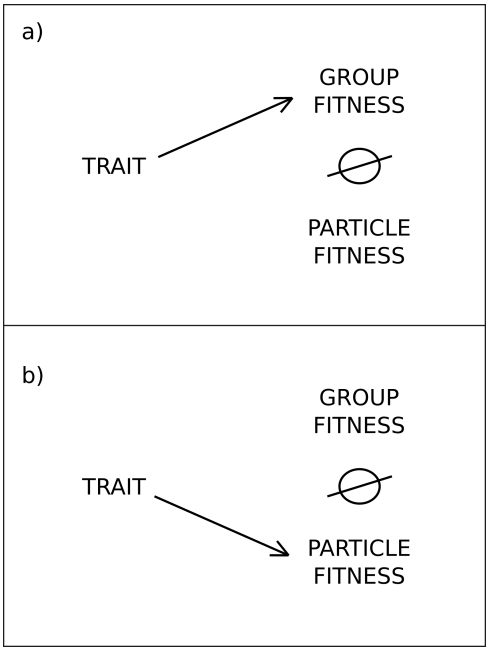


Fig 2. Emergent Relations. When there is no positive correlation between particle and group fitness, a trait-fitness relation at the group-level (a) is emergent; it cannot a by-product of a trait-fitness relation at the particle-level (b).

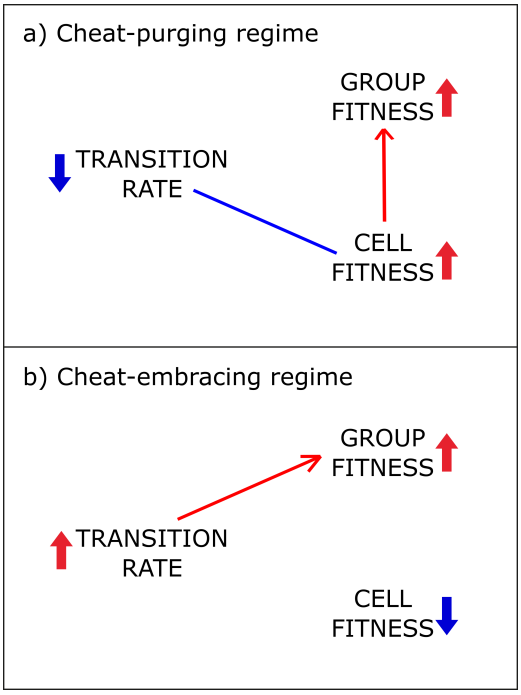
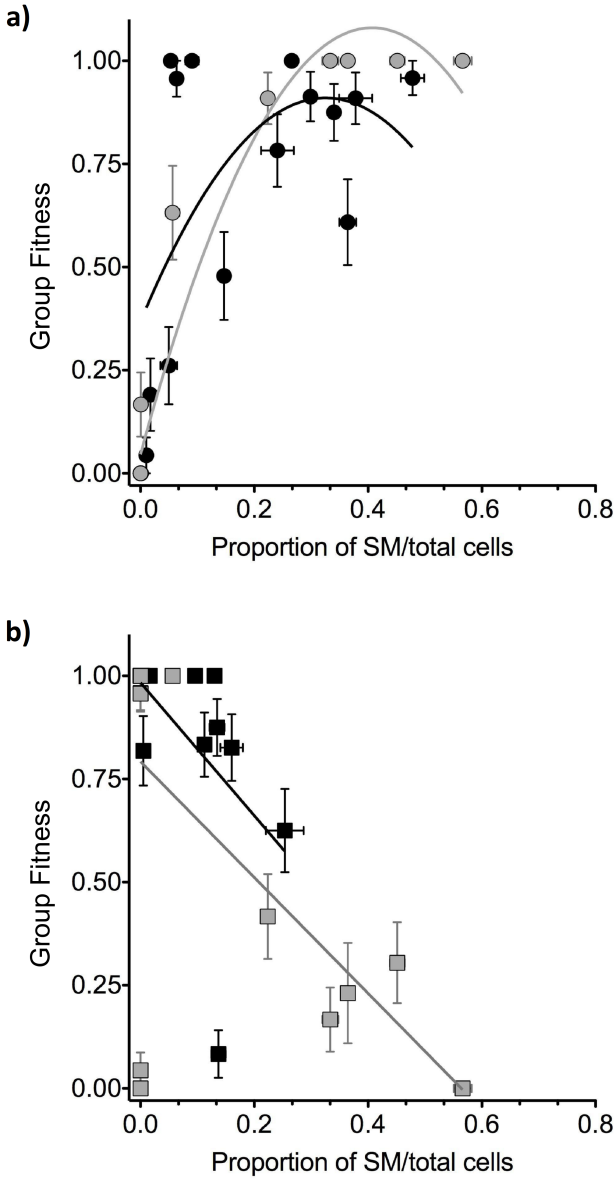


Fig 3. Detection of Emergent Relations in the Hammerschmidt et al. (2014) experiment. a) In the cheat-purging regime, the transition rate trait correlates with cell fitness and not group fitness: transition rate decreased and group fitness increased as a result of cell-level selection. b) In the cheat-embracing regime, there is no correlation between cell fitness and group fitness: there is an emergent relation between transition rate and group fitness. Small red and blue arrows represent an increase or decrease in the parameters indicated relative to ancestral lines. Long lines represent positive (red) or negative (blue) correlations, and long arrows indicate a likely causal relationship.



605

606 **Fig 4.** Relationship between particle composition and group fitness in the cheat-
607 embracing (a) and cheat-purging (b) regimes. Ancestral = grey, evolved = black.
608 Error bars are SEM, based on N=3.
609

610 **Supplementary Table**

611

612 Comparison of the fit of linear and quadratic models of the relationship between
613 the proportion of SM cells and group fitness in the cheat-embracing (CE) and
614 cheat-purging (CP) regimes using the Akaike's Information Criterion.

615 Prob correct: Probability that this model is the most likely to have generated the
616 data.

617

618

Parameters	**Difference in AICc**	**N**	**Prob Correct**	**df**	R^2
CE ancestral	151.5	360			
Linear model			**<0.01%**	358	0.712
Quadratic model			**>99.99%**	357	0.812
CE evolved	13.41	336			
Linear model			**0.12%**	334	0.128
Quadratic model			**99.88%**	333	0.167
CP ancestral	-1.747	360			
Linear model			**70.54%**	358	0.291
Quadratic model			**29.46%**	357	0.292
CP evolved	-1.689	360			
Linear model			**69.94%**	358	0.141
Quadratic model			**30.06%**	357	0.142

619