1 **Widespread density dependence of bacterial growth under acid stress**

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ABSTRACT

Benefits of cooperation intrinsically depend on density because biological interaction requires organismal proximity. Microbial cooperative traits are common, yet systematic tests for a shared cooperative phenotype across diverse species are rare, as are direct tests for the Allee effect - positive density dependence of fitness. Here we test for positive density dependence of growth under acid stress in five phylogenetically widespread bacterial species - three Gram-negative and two Gram-positive - and find the Allee effect in all five. However, social protection from acid stress appears to have evolved by different mechanisms across species. In *Myxococcus xanthus*, the acid-stress Allee effect is found to be mediated by pH-regulated secretion of a diffusible molecule present in supernatant from high-density cultures. In contrast, growth from low density under acid stress by the other species was not enhanced by high-density supernatant. Additionally, density dependence of *Myxococcus* fruiting-body formation during starvation is found to increase with acid stress, suggesting that abiotic stresses other than starvation shape the evolution of aggregative development. Our findings suggest that high cell density may protect against acid stress in most bacterial species and in *Myxococcus* may promote predation on microbes that acidify their local environment by secretion of metabolic byproducts.

Key words – Allee effect, eco-evo-devo, public goods, social evolution, stress response

INTRODUCTION

Organismal density often greatly affects survival and reproduction, both positively and negatively [1–4]. Such density dependence of fitness can mediate selection both on dispersive and aggregative traits that modify density *per se* and on other traits with density-sensitive fitness effects. A positive relationship between fitness and density - the Allee effect - is observed under some ecological conditions in many species [5–16].

Beneficial fitness effects of high density can be byproducts of simple organismal proximity. For example, organismal proximity can reduce heat or water loss, locomotion costs or predation risk [1,10,17–21]. Positive density-dependence (PDD) of fitness can also derive from social traits that are selectively favoured, at least in part, due to fitness benefits conferred on others by individuals expressing the focal trait [22,23]. Fitness benefits derived from conspecific interactions can be demonstrated experimentally by two distinct approaches. One approach is to demonstrate that interaction between individuals of distinct genotypes increase the fitness of a focal individual (or genotype) relative to its performance under identical conditions in the absence of inter-genotype interaction, even if total population density is held constant [24,25]. An alternative approach is to manipulate total population density and thereby demonstrate that average fitness increases with density [26,27].

Microbes express many traits known or hypothesized to benefit their conspecifics, including quorum-sensing signals that regulate gene-expression responses to environmental cues [28,29]. However, relatively few studies have directly demonstrated positive density-dependence (PDD) of population growth by manipulating cell density [7,30–32] with strains not artificially engineered to exhibit such PDD [28,33–35]. Fewer still have investigated relationships between density and fitness in microbial populations under stress imposed by fundamental abiotic environmental parameters such as pH, temperature and hydration [33,36–38], despite the major roles such forces have played in the evolution of microbial life. Here we experimentally examine the fitness response of several bacterial species to variable density across environments varying in pH, an important selective force across microbial habitats that varies greatly across both large and small spatio-temporal scales and strongly affects microbial community composition [39,40]. Acidity in particular is a common microbial stressor, and some individual bacterial species have been found to exhibit PDD of survival [41] or growth [30]

under acid stress. However, the phylogenetic generality of density-dependent growth under acid stress has not, to our knowledge, been tested systematically.

We first examine interactive effects of density and pH on growth by *Myxococcus xanthus*, a bacterium that exhibits unusually sophisticated cooperative traits among prokaryotes [42] and pervades terrestrial soils [43]. *M. xanthus* employs two distinct motility mechanisms with social components [44,45], preys on other microbial species [46] and forms multicellular fruiting bodies upon starvation [47,48]. The effectiveness of several *M. xanthus* behaviours is positively density dependent, including motility requiring Type IV pili, fruiting-body development, spore germination and growth on non-hydrolysed macropeptides [26,27,29,49,50]. *M. xanthus* predatory efficiency has also been hypothesized to depend positively on density [50].

After demonstrating that *M. xanthus* growth and development become increasingly dependent on density with increasing pH stress, we test whether such PDD of growth under acid stress is a common bacterial trait by examining four additional diverse bacterial species. We additionally investigate whether observed acid-stress Allee effects are mediated by diffusible “public good” secretions.

Methods

Strains

M. xanthus strain GJV1 is a direct descendant of the lab-reference strain DK1622, from which it differs by only several point mutations [51]. GJV1 is proficient at two *M. xanthus* motility systems - type IV-pili driven S-motility and A-motility, which can drive single-cell movement [44,52,53]. Strain DK3470 has a mutation that blocks production of extracellular matrix compounds required for cell-cell cohesion, full motility and development [54]. Strains of the Gram-positive species *Arthrobacter globiformis* (high-GC subdivision) and *Micrococcus luteus* (low-GC subdivision) and of the Gram-negative species *Pseudomonas fluorescens* (gamma subdivision) and *Rhizobium vitis* (alpha subdivision) used here are the same as those reported by Morgan *et al.* [55].

Media and culture conditions

For all species, CTT liquid (1% casitone; 8 mM MgSO₄; 10 mM Tris-HCl, pH 7.6; 1 mM potassium phosphate) [56] and CTT agar were used as growth media, agar plates were incubated at 32 °C and 90%

rH and liquid cultures were incubated at 32 °C while shaking at 300 rpm. For *M. xanthus* development assays, TPM liquid (identical to CTT liquid except lacking Casitone, the sole carbon source in CTT) and TPM agar (1.5%) plates were used. Media pH was adjusted with NaOH or HCl to the appropriate level before autoclaving. For *M. xanthus* growth cultures, samples from frozen stocks were inoculated and incubated CTT hard (1.5%) agar plates for three days. An inoculum from each plate was then transferred into eight ml CTT liquid in a 50 ml flask and grown overnight. These exponential-phase cultures were then transferred into larger volumes of CTT (culture/flask volume always 0.16) and allowed to grow further until populations exceeded the minimum requisite size. Cell densities of exponential-phase cultures were estimated with a TECAN Genios™ plate reader. Cultures were then centrifuged (5000 rpm, 15 min.) and resuspended to predicted densities of $\sim 1.25 \times 10^6$, 1.25×10^8 or 1.25×10^{10} cells/ml with CTT liquid. For other species, samples from frozen stocks were inoculated directly into CTT liquid.

Growth assays

OD (optical density)-based growth-rate assays reported in Fig. S1 were performed for *M. xanthus* strain GJV1. Cultures were grown in Greiner™ 6-well plates containing 5 ml of CTT agar per well, which was poured one day prior to inoculation and kept at room temperature overnight. Two wells of each plate were inoculated with 200 μ l of culture containing $\sim 2.5 \times 10^5$ (low density, LD), $\sim 2.5 \times 10^7$ (intermediate density, ID) or $\sim 2.5 \times 10^9$ (high density, HD) cells. Plates were gently rotated until the inoculated culture was spread evenly over the agar surface. The spread liquid cultures were then allowed to dry at room temperature in a laminar flow hood prior to capping the plates. OD values of each well were measured immediately after drying using a TECAN Genios™ plate reader and reflect the average of 16 reads per well in a 4 x 4 matrix. Further OD readings were taken every 12 h through 96 h. Three temporally separate biological replicates were performed with two technical replicates performed within each biological replicate. OD values from within-block technical replicates were averaged prior to statistical analysis across independent replicates. Maximum growth rate for each inoculation density x pH combination was calculated as the average slope of $\ln(\text{OD})$ across replicates for the 24-hour interval spanning the fastest average growth for the respective combination (Fig. S1).

Growth-rate assays with strain DK3470 were based on direct colony counts after dilution plating of harvested and dispersed cultures. 10 ml aliquots of CTT 1.5% agar at pH 5.0, 6.0 and 7.5 were poured into 50 ml Erlenmeyer flasks and allowed to solidify one day prior to the beginning of the growth assay. Flasks were kept covered at room temperature. 400 μ l of resuspended DK3470 culture containing approximately 1.25×10^6 (low density, LD), 1.25×10^8 (intermediate density, ID) or 1.25×10^{10} (high density, HD) cells/ ml were spread onto the agar surface and allowed to dry in a laminar flow hood for approximately 30 minutes. Due to destructive sampling, multiple flasks were prepared for each combination of pH and initial cell density. Immediately after the 30' drying period and after periods of incubation shown in Figs. 1 and S1, 1 ml of CTT liquid and ~10 glass beads (3 mm diameter) were placed in each flask and flasks were shaken for 10' at 300 rpm to disperse cells. Suspended cultures were then dilution plated into CTT 0.5% agar plates and resulting colonies were counted after incubation at 32 °C. Values shown in Fig. S1 indicate estimates of viable cell density in the 1-ml harvested suspensions.

For the four non-myxobacterial species, preliminary experiments identified acidic pH ranges at which growth in CTT liquid occurred but was substantially reduced in populations inoculated at a high initial density. Starting from initial densities (detailed below) differing by a factor of 10^4 , growth at a pH value in the relevant range for each species was subsequently analysed: *A. globiformis* at pH 4.35, *M. luteus* at pH 5.75, *P. fluorescens* at pH 4.6, and *R. vitis* at pH 4.25. Five ml of CTT 1.5% agar adjusted to the relevant pH for each species were poured into 6-well plate wells 24 hours prior to initiating growth assays. After agar solidified, a polyamide membrane filter (NL 16 Whatman™ membrane filters, 25 mm diameter, 0.2 μ m pore size) was placed on the agar surface of each well. Multiple wells were prepared for each replicate assay of each treatment because one filter was destructively sampled at each time point of each assay. Cultures were grown on filters in these assays because cells could not be easily harvested directly from the agar surface due to agar instability caused by acidity.

Cultures of each species were inoculated from freezer stocks and grown overnight in CTT liquid, centrifuged at 5000 rpm for 15 minutes and resuspended with CTT liquid to high density (HD, average cross-replicate densities of $\sim 1.6 \times 10^9$ cells/ml for *A. globiformis*, $\sim 5.8 \times 10^8$ cells/ml for *M. luteus*, ~ 1.3

$\times 10^9$ cells/ml for *P. fluorescens* and $\sim 1.7 \times 10^9$ cells/ml for *R. vitis* based on dilution plating immediately after resuspension) and diluted by a factor of 10^4 to generate low density (LD) cultures. 20 μ l aliquots of each HD and LD culture were spotted on filters and allowed to dry in a laminar flow hood. Plates were incubated at 32 °C and 90% rH. Immediately after spots were dry (t_0) and after 4, 8, 24, 28 and 32 hours, filters were removed and placed in a 50 ml flask containing 5 ml of CTT liquid and approximately 10 glass beads (3 mm diameter). Flasks were shaken at 300 rpm, 32 °C for 15 minutes to disperse cell populations, which were then dilution-plated into CTT 0.5% agar. Colonies were counted after 3-5 days of incubation at 32 °C, 90% rH.

Differences in the degree of growth between HD and LD populations at each timepoint N_t in the same replicate were calculated as: $\log_{10}(N_t(\text{HD})/N_0(\text{HD})) - \log_{10}(N_t(\text{LD})/N_0(\text{LD}))$.

Supernatant assays

M. xanthus: Following the same protocol as for the growth assay, we let *M. xanthus* cultures grow on CTT 1.5% agar in flasks at pH 6.0, 7.5 and 8.5 starting from low and high initial densities ($\sim 1.25 \times 10^6$ (LD) and $\sim 1.25 \times 10^8$ (ID) cells/ml, respectively). After 18 hours, we added 1 ml of CTT liquid to each flask and shook all flasks for 30 minutes at 300 rpm prior to collection of cell-free supernatant from each resuspended culture by filtration (0.2 μ m filters).

Cultures of *M. xanthus* growing in CTT liquid were subsequently spun down and resuspended to LD and ID with the six treatments of collected supernatants described above (derived from ID and LD cultures grown on agar at pH 6.0, 7.5 and 8.5) and CTT liquid as a control. 400 μ l of each resuspended culture were spread on CTT 1.5% agar adjusted to pH 6.0, 7.5 or 8.5. After 18 hours of incubation, 1 ml of CTT liquid and ~ 10 glass beads (3 mm diameter) were added to each flask and flasks were shaken for 10' at 300 rpm prior to dilution plating to obtain colony counts.

Non-myxobacteria: Non-myxobacterial species were inoculated in liquid CTT media, incubated overnight at 32 °C at 300 rpm, centrifuged at 5000 rpm for 15 min and resuspended in CTT liquid to the respective high-density (HD) value for each species and then diluted by a factor of 10^4 to generate the respective low-density (LD) treatment. 20 μ l aliquots of these HD and LD cultures were then individually spotted on a polyamide membrane filter placed on CTT 1.5% agar with pH adjusted to

both pH 7.5 (control) and the same acidic pH value used for the acid-stress growth assays described above and the resulting cultures were incubated for 18 hrs. To generate supernatant for the next step of the experiment, filters were then removed from the agar beds, placed in 1 ml CTT liquid, incubated at 100 rpm on a gel rocker for 30 minutes, and the filter-treated CTT liquid was then filter sterilized using a 0.2 μ m filter.

The resulting culture supernatants were used to then resuspend LD and HD cultures of the respective non-myxobacterial species, and growth assays were performed to measure the influence of diffusible substances on the growth of LD and HD cultures. To do so, cultures of *A. globiformis*, *M. luteus*, *R. vitis* and *P. fluorescens* that had been incubated in CTT liquid for 48 hours were spun down and resuspended to HD and LD using the supernatant derived from either HD or LD cultures growing at either the respective acid-stress pH or at pH 7.5, as described above. 20 μ l of these resuspended cultures were then spotted on a filter placed on CTT agar at the respective acidic pH for each species and additional samples from the same resuspended cultures were dilution-plated to obtain T0 population-size estimates. Plated samples were then incubated for 18 hrs. To obtain population-size estimates at T18, filters were placed in 12-well-plate wells containing 1 ml CTT liquid and cultures were dispersed by repeated pipetting prior to dilution plating.

Fruiting-body development

Development assays were performed in Greiner™ 6-well plates containing 5 ml pH-adjusted TPM 1.5% agar per well, which was poured one day prior to inoculation and kept at room temperature overnight. Vegetatively growing cultures of GJV1 were centrifuged, resuspended in TPM liquid to $\sim 5 \times 10^9$ cells/ml and subsequently diluted into two lower density treatments of $\sim 1 \times 10^9$ and $\sim 3 \times 10^8$ cells/ml. Ten μ l aliquots for each density treatment were placed on TPM agar and allowed to dry in a laminar flow hood. After five days of incubation, developmental phenotypes were photographed. Three temporally independent replicate assays were performed for each density-pH combination with two technical replicates performed in adjacent wells within each independent replicate.

Results

Myxococcus survival and growth under pH stress is strongly density dependent

Early experiments with the reference strain GJV1 growing on agar surfaces that measured optical density (OD) over time suggested that growth rate was density dependent across a wide range of pH levels, but also that the degree of density dependence might be greater under more acidic and more basic conditions than at more benign pH levels (Fig. S1). Especially in the acidic direction, growth rate appeared more sensitive to deviation from pH 7.5 (at which the lowest-density populations grew best) at lower cell density, although greatly increased experimental error at the most extreme pH values obscured estimates in those treatments. To facilitate more direct estimates of population size, we further investigated the effect of acid stress on density-dependent growth by *M. xanthus* using strain DK3470, which disperses more easily in liquid after growth on agar than GJV1 due to a mutation that reduces production of extracellular matrix components [57,58]. Based on the results with GJV1, we performed growth assays with DK3470 at pH 5.0, 6.0 and 7.5.

When cells were inoculated onto CTT agar at low density (LD, $\sim 1.25 \times 10^6$ cells/ml) at the three pH levels, viable population size decreased in both acidic treatments (pH 5.0, 6.0) until no viable cells were detected at later time points, whereas populations grew robustly from the same low density at pH 7.5 (Figs. 1, S2). Starting at 100-fold higher density (intermediate density, ID, $\sim 1.25 \times 10^8$ cells/ml), viable populations decreased to non-detection at pH 5.0 but grew steadily at both pH 6 and pH 7.5. Populations initiated at the highest density (HD, $\sim 1.25 \times 10^{10}$ cells/ml) increased only slightly before reaching maximum population size. However, these HD populations reveal positive density dependence of survival in that they did not decrease substantially even at pH 5.0 as did the lower-density populations, but rather remained relatively stable throughout the experiment in all pH treatments. Thus, under even mild acid stress, cell density determines whether *M. xanthus* populations survive and grow. These outcomes reflect a strong Allee effect [6] in which populations increase or decrease depending on whether they are above or below a threshold density, respectively.

A pH-regulated diffusible secretion mediates PDD of M. xanthus growth under acid stress

We tested whether the exported social molecule(s) mediating density-dependent growth of *M. xanthus* under acid stress is diffusible or cell-bound. To do so, we supplemented populations initiated at LD and pH 6.0 - conditions that normally lead to population decline (Fig. 1) - with filter-sterilized supernatant from cultures that had been initiated at LD or ID (same densities as in the Fig. 1 experiment) and incubated at pH 6.0, 7.5 and 8.5 for 18 h. As expected, supernatant from all populations initiated at LD, regardless of the pH at which those populations were incubated, failed to prevent population decline of cultures initiated at LD and pH 6.0, as did control supplementation with CTT growth medium (Fig. 2). However, supernatant from both ID populations incubated at the two lower pH levels induced significant growth of LD populations at pH 6. These outcomes demonstrate that density-dependent survival and growth by *M. xanthus* is mediated by a diffusible secretion. Unlike supernatant derived from ID cultures grown at pH 6.0 and 7.5, supernatant from ID cultures growing at pH 8.5 failed to induce growth of LD, pH 6.0 cultures. This latter outcome reveals that the production and/or stability of the diffusible *M. xanthus* PDD factor is regulated by environmental pH.

The secreted PDD factor might protect from acid toxicity either by increasing environmental pH or by inducing changes in cellular state that render cells less sensitive to unchanged acidity. To test between these possibilities, we compared the pH of supernatant from ID, pH 6.0 cultures after 18 hrs of incubation to pH measurements of the original medium but found no significant change ($p > 0.05$, one sample *t*-test). This outcome indicates that the secreted PDD factor exerts its protective effects by a mechanism other than altering environmental pH.

PDD of aggregative fruiting body development increases with acid stress

In light of the acid-stress Allee effects on vegetative growth documented above, we asked whether PDD of *M. xanthus* fruiting body development already known to occur at benign pH [26,29] increases in strength with increasing acid stress. Populations of the developmentally proficient strain GJV1 were incubated on TPM starvation agar at five pH levels (pH 5.5, 6.0, 6.5, 7.0 and 7.5) and three initial cell densities (HD, ID and LD, see Methods) and developmental phenotypes were observed. While dark fruiting bodies formed at all five pH conditions in the HD treatment, the degree of developmental

aggregation was found to be more sensitive to acid stress at lower densities (Fig. 3). ID populations showed only very slight aggregation at the lowest pH and LD populations did not even minimally aggregate at the two lowest pH levels. Thus, the negative effect of decreasing cell density on developmental aggregation is greater under acid stress than at benign pH levels.

Acid-stress Allee effects are common across the eubacteria domain but appear to be mediated by diverse mechanisms

Our results with *M. xanthus* together with results from prior studies [30,59,60] led us to ask whether acid-stress Allee effects are common across the eubacteria domain. Pilot experiments identified acidic pH ranges at which growth of four non-myxobacterial species - *Arthrobacter globiformis*, *Micrococcus luteus*, *Pseudomonas fluorescens* and *Rhizobium vitis* - still occurred at least at high density but was reduced relative to less acidic pH. Subsequently, at one pH level for each species at which high-density growth was reduced, changes in population size by all four species over a 32 h period were measured in cultures initiated from two starting densities differing by a factor of 10^4 (see Methods). PDD of growth under acid stress was clearly evident in all four species (Figs. 4, S3). In three species (*A. globiformis*, *P. fluorescens* and *R. vitis*), the LD populations decreased while the HD populations increased (strong Allee effects), while in *M. luteus* the HD populations increased more than did LD populations (a weak Allee effect)(Fig. S3).

To examine whether acid-stress Allee effects are mediated by similar mechanisms across bacterial species, we tested whether, as occurs in *M. xanthus* (Fig. 2), supernatant from HD cultures of the four non-myxobacterial species mitigates the disadvantage of LD cultures subjected to acid stress relative to HD cultures. HD cultures treated with any supernatant consistently performed better under acid stress in these experiments than the respective LD cultures treated with the same supernatant (Fig. S4), either remaining stable or declining less than the corresponding LD cultures, which consistently decreased significantly due to acid stress. However, unlike HD supernatant from *M. xanthus*, HD supernatants from the other species did not improve the performance of acid-stressed LD cultures (Fig. S4). This result suggests that the Allee effects observed in these species are mediated either by i) cell-bound rather than diffusible molecules or ii) diffusible molecules that rapidly diffuse away into agar after cultures

were spotted onto experimental plates and thus have little protective effect under our experimental conditions.

Discussion

Molecules mediating social interactions play major roles in microbial life histories [e.g. 2,22,23,25,44,45,47,61,62]. Having found that social molecules greatly mitigate acid stress by the complexly social bacterium *M. xanthus*, we investigated whether social protection from toxic acidity is a widespread trait among prokaryotes. Indeed, all five phylogenetically diverse species examined here exhibited clear positive density-dependence of growth under acid stress. Moreover, additional examples of social protection from pH stress have previously been reported in yet other individual species [30,38,59,63,64]. Collectively with those earlier reports, our phylogenetically broad experiments suggest that social forms of protection from toxic acidity are the norm rather than the exception among eubacteria.

Yet despite the prevalence of social protection from acid stress across bacteria, this phenotype has clearly evolved by diverse molecular mechanisms. *Streptococcus mutans* cells protect themselves against oral-cavity acidity (that they themselves increase) by secreting a quorum-sensing peptide that in turn triggers stress-protective physiological change [38]. *Corynebacterium ammoniagenes* eliminates toxic acidity in urea-containing medium by secreting urease [30]. *M. xanthus* does not appear to significantly alter its environmental pH (at least under our experimental conditions), but does secrete a protective diffusible factor. The four non-myxobacterial species examined here, however, appear to employ non-diffusible protective molecules (or diffusible protectants of a different character than that of *M. xanthus*), as supernatant from high-density cultures of these species did not protect acid-stressed low-density populations from population decline, as occurred in *M. xanthus*. Thus, just drawing from our own experiments and the examples of *S. mutans* and *C. ammoniagenes*, different forms of bacterial social protection against acid stress appear to involve both diffusible and non-diffusible factors and to involve both predominantly cell-level protection and environmental modification. More detailed investigations of the species examined here and other species are likely to reveal yet greater mechanistic diversity.

High conspecific cell density is the norm for microbes growing as colonies in structured habitats, for example in biofilms [38,65,66], and is thus expected to be a major selective force in most natural contexts, including under both stressful and benign conditions. To fully understand the social lives of microbes, fitness effects of density both i) within stressful ranges of diverse abiotic parameters such as hydration level, oxygen level, pH, salinity, starvation, and temperature and ii) while subject to biotic antagonisms such as interference competition [28], immunity [31] and predation [7] need to be quantified across a broad range of microbes and the molecular mechanisms of discovered Allee effects and their evolutionary causes correspondingly investigated. Intriguingly, the yeast *Saccharomyces cerevisiae* has recently been found to socially protect itself from temperature stress by secretion of glutathione [33]. Costly production of such abiotic-stress protectants should often be cheatable by non-producing or low-producing genotypes [27]. Thus, genetic diversity among conspecifics for variation at protectant-production levels due to such exploitation is expected to be common.

Considering the myxobacteria, traits previously shown to depend positively on cell density have been associated, whether directly or indirectly, with motility [67], aggregative fruiting-body development [26,68] or predation [50] - the social behaviours for which the myxobacteria are best known. Additionally, due to strong positive frequency-dependence of interference competition between natural isolates of *M. xanthus*, high local density should also promote competitiveness in direct cell-cell combat when diverged cell groups meet in the soil [69]. Here we show that simple vegetative growth by *M. xanthus* under acid stress is dependent on a social trait. Social resistance to pH stress appears to be independent of other complex *M. xanthus* social behaviours, as it was documented most extensively here using a strain defective at TFP-based motility and aggregative development.

These findings with *M. xanthus* also have implications for understanding microbial “eco-evo-devo” [70], or how ecological conditions shape the evolution of microbial developmental systems. We found that acid stress amplifies dependence of *M. xanthus* fruiting-body development on cell density beyond that observed at benign pH [26,29]. Specifically, the minimum cell density required to allow developmental aggregation increases with acid stress (Fig. 3). This suggests that stressfully acidic natural conditions select more strongly for traits promoting high local density upon nutrient depletion than do less stressful pH conditions. Thus, acid stress and potentially other forms of abiotic stress may

impact how the relationship between density and development (Fig. 3) - including output of spores that subsequently germinate and grow - evolves. Such environmental stresses may thereby select on density-determining traits such as motility behaviour and adhesin production, not only in the myxobacteria but in other aggregative developmental systems as well.

In the myxobacteria, spores are often formed within fruiting bodies. Hypothesized benefits of sporulating within elevated, dense groups rather than more individualistically outside of fruiting bodies include increased probability of dispersal to growth-conducive environments and increased spore quality and survival [2,42,71]. Another idea proposes that the high density of spores within fruiting bodies enhances germination and subsequent growth after encountering nutrient levels conducive to growth [42,50,72]. Consistent with the latter hypothesis, spore germination was recently found to depend positively on density under both benign conditions and under saline stress [27]. Our results suggest that tight packing of spores benefits spore germination and subsequent growth if fruiting bodies encounter acidic conditions.

Microbes often modify environmental pH and in so doing can strongly impact microbial interactions [30]. For example, *Escherichia coli* generates acidic byproducts from metabolizing glucose, which has been shown to inhibit host colonization by *Vibrio cholerae* [73]. Our findings suggest that environmental acidification by potential prey may make myxobacterial predatory fitness density-dependent by a different mechanism than previously proposed. Earlier experiments showed that the growth rate of *M. xanthus* liquid cultures on large macromolecules that require extracellular hydrolysis for optimal metabolic use is density dependent, presumably because higher concentrations of extracellular enzymes in high density cultures facilitate more rapid access to hydrolyzed peptides [50]. This has often been cited as evidence that myxobacterial predation efficiency is likely to also be density dependent, although the evidence for this hypothesis has remained indirect. Our results suggest that prey metabolism rather than (or in addition to) predator metabolism may cause predation-fueled growth of myxobacteria to be density dependent when myxobacteria encounter prey colonies that have acidified their local environment.

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AUTHOR CONTRIBUTIONS

All authors designed experiments, analysed data and drafted manuscript components. FF, SP and HP performed experiments; and FF, SP and GJV revised the manuscript. HP performed early experiments that first revealed effects of density on *M. xanthus* growth and development under acid stress.

REFERENCES

1. Ruxton GD, Sherratt TN. 2006 Aggregation, defence and warning signals: The evolutionary relationship. *Proc. R. Soc. B.* **273**, 2417–2424.
2. Velicer GJ, Vos M. 2009 Sociobiology of the myxobacteria. *Annu. Rev. Microbiol.* **63**, 599–623.
3. Alexander RD. 1974 The evolution of social behaviour. *Annu. Rev. Ecol. Syst.* **5**, 325–383.
4. Arneberg P, Skorping A, Grenfell B, Read AF. 1998 Host densities as determinants of abundance in parasite communities. *Proc. R. Soc. B* **265**, 1283–1289.
5. Allee WC, Emerson AE, Park O, Park T, Schmidt KP. 1949 Principles of Animal Ecology. W. B. Saunders Co., Philadelphia and London.
6. Berec L, Angulo E, Courchamp F. 2007 Multiple Allee effects and population management. *Trends Ecol. Evol.* **22**, 185–191.
7. Kaul RB, Kramer AM, Dobbs FC, Drake JM. 2016 Experimental demonstration of an Allee effect in microbial populations. *Biol. Lett.* **12**, 20160070.
8. Stephens PA, Sutherland WJ, Freckleton RP. 1999 What is the Allee effect? *Oikos* **87**, 185–190.
9. Courchamp F, Berec L, Gascoigne G. 2008 Allee effects in ecology and conservation. Oxford University Press, New York.
10. Williams CK, Lutz RS, Applegate RD. 2003 Optimal group size and northern bobwhite coveys. *Anim. Behav.* **66**, 377–387.

11. Higashi M, Yamamura N. 1003 What determines animal group size? Insider-outsider conflict and its resolution. *Am. Nat.* **142**, 553-563.
12. Vander Wall SB, Enders MS, Waitman BA. 2009 Asymmetrical cache pilfering between yellow pine chipmunks and golden-mantled ground squirrels. *Anim. Behav.* **78**, 555–561.
13. Pedersen B, Hanslin HM, Bakken S. 2001 Testing for positive density-dependent performance in four bryophyte species. *Ecology* **82**, 70–88.
14. Cresswell W. 1994 The function of alarm calls in redshanks, *Tringa totanus*. *Anim. Behav.* **47**, 736–738.
15. Sherman PW. 1977 Nepotism and the evolution of alarm calls. *Science* **197**, 1246–1253.
16. Clark BR, Faeth SH. 1998. The evolution of egg clustering in butterflies: A test of the egg desiccation hypothesis. *Evol. Ecol.* **112**, 543–552.
17. Potts M. 1994 Desiccation tolerance of prokaryotes. *Microbiol. Rev.* **58**, 755–805.
18. Bill RG, Herrnkind W. 1976 Drag reduction by formation movement in spiny lobsters. *Science* **193**, 1146-1148.
19. Sillen-Tullberg B, Leimar O. 1988 The evolution of gregariousness in distasteful insects as a defense against predators. *Am. Nat.* **132**, 723–734.
20. Turner G. Pitcher TJ. 1986 Attack abatement: a model for group protection by combined avoidance and dilution. *Am. Nat.* **128**, 228-240.
21. Pulliam HR. 1973 On the advantages of flocking. *J. Theor. Biol.* **38**, 419–422.
22. Brockhurst MA, Habets MGJL, Libberton B, Buckling A, Gardner A. 2010 Ecological drivers of the evolution of public-goods cooperation in bacteria. *Ecology* **91**, 334–340.
23. Darch SE, West SA, Winzer K, Diggle SP. 2012 Density-dependent fitness benefits in quorum-sensing bacterial populations. *Proc. Natl Acad. Sci. USA* **109**, 8259–8263.
24. Velicer GJ, Kroos L, Lenski RE. 2000 Developmental cheating in the social bacterium *Myxococcus xanthus*. **404**, 598-601.
25. Pollak S, Omer-Bendori S, Even-tov E, Lipsman V, Bareia T, Ben-Zion I, et al. 2016 Facultative cheating supports the coexistence of diverse quorum-sensing alleles. *Proc. Natl Acad. Sci. USA* **113**, 2152–2157.

26. Kadam SV, Velicer GJ. 2006 Variable patterns of density-dependent survival in social bacteria. *Behav. Ecol.* **17**, 833–838.
27. Pande S, Pérez Escriba P, Yu YTN, Sauer U, Velicer GJ. 2020 Cooperation and cheating among germinating spores. *Curr. Biol.* **30**, 4745–4752.
28. Maldonado-Barragán A, West SA. 2020 The cost and benefit of quorum sensing-controlled bacteriocin production in *Lactobacillus plantarum*. *J. Evol. Biol.* **33**, 101–111.
29. Kaplan HB, Plamann L. 1996 A *Myxococcus xanthus* cell density-sensing system required for multicellular development. *FEMS Microbiol. Lett.* **139**, 89–95.
30. Ratzke C, Gore J. 2018 Modifying and reacting to the environmental pH can drive bacterial interactions. *PLoS Biol.* **16**, e2004248.
31. Landsberger M, Gandon S, Meaden S, Rollie C, Chevallereau A, Chabas H, et al. 2018 Anti-CRISPR phages cooperate to overcome CRISPR-Cas immunity. *Cell* **174**, 908–916.
32. Andreu-Moreno I, Sanjuán R. 2020 Collective viral spread mediated by virion aggregates promotes the evolution of defective interfering particles. *mBio* **11**, e02156-19.
33. Laman Trip DS, Youk H. 2020 Yeasts collectively extend the limits of habitable temperatures by secreting glutathione. *Nat. Microbiol.* **5**, 943–954.
34. Dressler MD, Conde J, Eldakar OT, Smith RP. 2019 Timing between successive introduction events determines establishment success in bacteria with an Allee effect. *Proc. R. Soc. B* **286**, 20190598.
35. Smith R, Tan C, Srimani JK, Pai A, Riccione KA, Song H, You L. 2014 Programmed Allee effect in bacteria causes a tradeoff between population spread and survival. *Proc. Natl Acad. Sci. USA* **111**, 1969–1974.
36. Wilson M, Lindow SE. 1994 Inoculum density-dependent mortality and colonization of the phyllosphere by *Pseudomonas syringae*. *Appl. Environ. Microbiol.* **60**, 2232–2237.
37. Monier JM, Lindow SE. 2003 Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. *Proc. Natl Acad. Sci. USA* **100**, 15977–15982.
38. Li YH, Hanna MN, Svensater G, Ellen RP, Cvitkovitch DG. 2001 Cell density modulates acid adaptation in *Streptococcus mutans*: Implications for survival in biofilms. *J. Bacteriol.* **183**, 6875–6884.

39. Bardgett R. 2005 The biology of soil: A community and ecosystem approach. *In* The biology of soil. Oxford University Press.
40. Hartman WH, Richardson CJ, Vilgalys R, Bruland GL. 2008 Environmental and anthropogenic controls over bacterial communities in wetland soils. *Proc. Natl Acad. Sci. USA* **105**, 17842–17847.
41. D’Souza C, Alva PP, Padyana AK, Karunasagar I, Kumar BK. 2019 Unveiling the acid stress response of clinical genotype *Vibrio vulnificus* isolated from the marine environments of Mangaluru coast, India. *Can. J. Microbiol.* **65**, 681-690.
42. Shimkets LJ, Dworkin M, Reichenbach H. 2006 The myxobacteria. *In* The Prokaryotes 7, 31–115. Springer, New York, NY.
43. Dawid W. 2000 Biology and global distribution of myxobacteria in soils. *FEMS Microbiol. Rev.* **24**, 403–427.
44. Wu SS, Kaiser D. 1995 Genetic and functional evidence that Type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol. Microbiol.* **18**, 547–558.
45. Jakobczak B, Keilberg D, Wuichet K, Sogaard-Andersen L. 2015 Contact- and protein transfer-dependent stimulation of assembly of the gliding motility machinery in *Myxococcus xanthus*. *PLoS Genet.* **11**, e1005341.
46. Berleman JE, Kirby JR. 2009 Deciphering the hunting strategy of a bacterial wolfpack. *FEMS Microbiol. Rev.* **33**, 942-957.
47. Shimkets LJ. 1999 Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. *Annu. Rev. Microbiol.* **53**, 525–549.
48. Jelsbak L, Sogaard-Andersen L. 2000 Pattern formation: Fruiting body morphogenesis in *Myxococcus xanthus*. *Curr. Opin. Microbiol.* **3**, 637–642.
49. Rosenbluh A, Nir R, Sahar E, Rosenberg E. 1989 Cell-density-dependent lysis and sporulation of *Myxococcus xanthus* in agarose microbeads. *J. Bacteriol.* **171**, 4923–4929.
50. Rosenberg E, Keller KH, Dworkin M. 1977 Cell density-dependent growth of *Myxococcus xanthus* on casein. *J. Bacteriol.* **129**, 770–777.

51. Velicer GJ, Raddatz G, Keller H, Deiss S, Lanz C, Dinkelacker I, Schuster SC. 2006 Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. *Proc. Natl Acad. Sci. USA* **103**, 8107–8112.
52. Sun M, Wartel M, Cascales E, Shaevitz JW, Mignot T. 2011 Motor-driven intracellular transport powers bacterial gliding motility. *Proc. Natl Acad. Sci. USA* **108**, 7559–7564.
53. Rodriguez AM, Spormann AM. 1999 Genetic and molecular analysis of *cglB*, a gene essential for single-cell gliding in *Myxococcus xanthus*. *J. Bacteriol.* **181**, 4381–4390.
54. Dana JR, Shimkets LJ. 1993 Regulation of cohesion-dependent cell interactions in *Myxococcus xanthus*. *J. Bacteriol.* **175**, 3636–3647.
55. Morgan AD, Maclean RC, Hillesland KL, Velicer GJ. 2010 Comparative analysis of *Myxococcus* predation on soil bacteria. *Appl. Environ. Microbiol.* **76**, 6920–6927.
56. Vos M, Velicer GJ. 2006 Genetic population structure of the soil bacterium *Myxococcus xanthus* at the centimeter scale. *Appl. Environ. Microbiol.* **72**, 3615–3625.
57. Shimkets LJ. 1986 Role of cell cohesion in *Myxococcus xanthus* fruiting body formation. *J. Bacteriol.* **166**, 842–848.
58. Yang Z, Ma X, Tong L, Kaplan HB, Shimkets LJ, Shi W. 2000 *Myxococcus xanthus dif* genes are required for biogenesis of cell surface fibrils essential for social gliding motility. *J. Bacteriol.* **182**, 5793–5798.
59. Matsui R, Cvitkovitch D. 2010 Acid tolerance mechanisms utilized by *Streptococcus mutans*. *Future Microbiol.* **5**, 403–417.
60. Mates AK, Sayed AK, Foster JW. 2007 Products of the *Escherichia coli* acid fitness island attenuate metabolite stress at extremely low pH and mediate a cell density-dependent acid resistance. *J. Bacteriol.* **189**, 2759–2768.
61. Lazazzera BA. 2000 Quorum sensing and starvation: Signals for entry into stationary phase. *Curr. Opin. Microbiol.* **3**, 177–182.
62. Redfield RJ. 2002 Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol.* **10**, 365–370.

63. Cvitkovitch DG, Li YH, Ellen RP. 2003 Quorum sensing and biofilm formation in Streptococcal infections. *J. Clin. Invest.* **112**, 1626-1632.
64. Loesche WJ. 1986 Role of *Streptococcus mutans* in human dental decay. *Microbiol. Mol. Biol. Rev.* **50**, 353–380.
65. Li YH, Tang N, Aspiras MB, Lau PCY, Lee JH, Ellen RP, *et al.* 2002 A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J. Bacteriol.* **184**, 2699–2708.
66. Fux CA, Costerton JW, Stewart PS, Stoodley P. 2005 Survival strategies of infectious biofilms. *Trends Microbiol.* **13**, 34–40.
67. Berleman JE, Vicente JJ, Davis AE, Jiang SY, Seo YE, Zusman DR. 2011 FrzS regulates social motility in *Myxococcus xanthus* by controlling exopolysaccharide production. *PLoS One* **6**, e23920.
68. Kuspa A, Plamann L, Kaiser D. 1992 A-signalling and the cell density requirement for *Myxococcus xanthus* development. *J. Bacteriol.* **174**, 7360–7369.
69. Rendueles O, Amherd M, Velicer GJ. 2015 Positively frequency-dependent interference competition maintains diversity and pervades a natural population of cooperative microbes. *Curr. Biol.* **25**, 1673-1681.
70. Abouheif E, Favé MJ, Ibarrarán-Viniegra AS, Lesoway MP, Rafiqi AM, Rajakumar R. 2014 Eco-Evo-Devo: The Time Has Come. In Landry C., Aubin-Horth N (eds). Ecological Genomics. Advances in Experimental Medicine and Biology, vol 781. Springer, Dordrecht.
71. La Fortezza M, Schaal KA, Velicer GJ. In press. Group formation: On the evolution of aggregative multicellularity. Chapter 6 in Herron MD, Conlin PL, Ratcliff WC (eds). The Evolution of Multicellularity. CRC Press, Boca Raton, FL.
72. Kaiser D. 2001 Building a multicellular organism. *Annu. Rev. Genet.* **35**, 103–123.
73. Nag D, Breen P, Raychaudhuri S, Withey JH, and Freitag NE. 2018 Glucose metabolism by *Escherichia coli* inhibits *Vibrio cholerae* intestinal colonization of Zebrafish. *Infect. Immun.* **86**, e00486-18.

Figure Legends

Fig. 1. High density allows growth of *M. xanthus* under acid stress that kills low-density populations. Log-transformed ratios of population size (N_t) of *M. xanthus* strain DK3470 over time relative to starting size (N_0) are shown for populations inoculated in media of pH 5.0 (red shading), 6.0 (blue) or 7.5 (green) at high (HD), intermediate (ID) and low (LD) density (see Methods). Shading represents 95% confidence regions ($n = 3$).

Fig. 2. *M. xanthus* growth under acid stress is mediated by a diffusible public good. Supernatants from intermediate-density (ID) cultures of DK3470 grown at pH 6.0 or 7.5 protect low-density (LD) populations from acid stress (pH 6.0) and thereby allow population growth (green circles). Control media (CTT), supernatants from LD cultures grown at three tested pH values and supernatants from ID cultures grown under basic conditions (pH 8.5) do not protect LD cultures from population crashes caused by acid stress (red circles). Error bars represent 95% confidence intervals ($n = 3$).

Fig. 3. Strength of PDD during aggregative multicellular development by *M. xanthus* increases with acid stress. Developmental phenotypes after five days of starvation for populations initiated at three cell densities (HD, $\sim 5 \times 10^9$ cells/ml; ID, $\sim 10^9$ cells/ml; LD, $\sim 3 \times 10^8$ cells/ml) and five pH values are shown. Dark spots are fruiting bodies in which many light-refractive spores have formed whereas light spots are aggregates with fewer, if any, stress-resistant spores.

Fig. 4. Growth under acid stress depends on density across highly diverged bacterial species. Growth differences between high-density (HD) vs low-density (LD) populations of four bacterial species under acid stress are shown. Values greater than 0 indicate that the respective HD populations increased more than the respective LD populations by the relevant time point. The y-axis unit reflects a ten-fold difference in the degree of population-size change from the start of the experiment. (See also Fig. S3.) Shaded areas represent 95% confidence regions ($n = 3$).

Fig. S1. Maximum growth-rate estimates for GJV1 populations measured as highest growth over a 24-h period from OD-based growth measurements on CTT agar at nine pH levels. LD, ID and HD populations are represented by squares, circles and triangles, respectively. Colours indicate acidic, near-neutral and basic pH ranges (red, green and blue, respectively). Error bars represent 95% confidence intervals ($n = 3-4$).

Fig. S2. DK3470 $\log_{10}(\text{CFU/ml})$ for HD, ID and LD populations incubated at pH 7.5 (green), 6.0 (blue) and 5.0 (red). Error bars represent 95% confidence intervals ($n = 3$).

Fig. S3. $\log_{10}(\text{CFU/ml})$ values for HD and LD populations of *A. globiformis*, *M. luteus*, *P. fluorescens*, and *R. vitis* incubated under acid stress for 32 h. Error bars represent 95% confidence intervals ($n = 3$).

Fig. S4. The disadvantage of acid-stressed low-density populations of non-myxobacterial species is not mitigated by supernatant from either high- or low-density (HD or LD, respectively) cultures grown at either acidic pH or pH 7.5 or by control media (CTT). A. Log-ratio of population size after vs before 18 h incubation under acid stress with the specified supernatant treatment or control. Solid and open circles represent cultures initiated at HD and LD, respectively, that were treated with supernatant from cultures grown under the density and pH conditions specified below the graphs. Recipient populations were incubated at the same acidic pH as in the experiment reported in Figs. 4 and S3. Letters indicate statistically distinct mean sets (Tukey's posthoc test $p < 0.05$). B. Differences of log-ratios presented in Fig. S4A between LD vs HD populations. Grey circles represent statistically significant differences in population-size change between LD cultures vs HD cultures resuspended in the same culture supernatant (or CTT control)(one-sample t -test for significant differences relative to 0, multiple-test-corrected $p < 0.01$). Error bars represent 95% confidence intervals ($n = 3$).

553 **Figure 1**

554 **Figure 2**

555 **Figure 3**

556 Figure 4

557 **Figure S1**

558 **Figure S2**

559 Figure S3