Hidden paths to endless forms most wonderful: Ecology latently shapes evolution of multicellular development in predatory bacteria

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SUMMARY

Ecological causes of developmental-system evolution, for example from predation, remain under intense investigation. An important open question is the role of latent phenotypes in eco-evo-devo. The predatory bacterium *Myxococcus xanthus* undergoes aggregative multicellular development upon starvation. Here we use *M. xanthus* to test whether evolution in several distinct growth environments that do not induce development latently alters developmental phenotypes, including morphology and plasticity, in environments that do induce development. In the MyxoEE-3 evolution experiment, growing *M. xanthus* populations swarmed across agar surfaces while adapting to distinct conditions varying at factors such as surface stiffness or prey identity. All examined developmental phenotypes underwent extensive and ecologically specific latent evolution, with surface stiffness, prey presence and prey identity all strongly impacting the latent evolution of development. Evolution on hard agar allowed retention of developmental proficiency and extensive stochastic phenotypic radiation, including of reaction norms, with instances of both increased plasticity and canalization. In contrast, evolution on soft agar latently led to systematic loss of development, revealing an ecologically-contingent fitness trade-off between the growth and developmental phases of a multicellular life cycle that is likely determined by details of motility behavior. Similar contingency was observed after evolution during predatory growth in distinct prey environments, with *Bacillus subtilis* causing greater loss of development and lower stochastic diversification than *Escherichia coli.* Our results have implications for understanding evolutionary interactions among predation, development and motility in myxobacterial life cycles, and, more broadly, the importance of latent phenotypes for the diversification of developmental systems.

Keywords: aggregative development, eco-evo-devo, trade-offs, life-history theory, multicellularity, pleiotropy, predator-prey interactions

INTRODUCTION

Ecological context fundamentally shapes the remarkable diversification[1] of developmental programs, a notion that precedes the modern field of evolutionary developmental biology, or evo-devo[2]. A recently-emerged focus on eco-evo-devo seeks to quantitatively understand such ecological causation of developmental evolution[3,4]. Many biotic ecological factors, such as predator-prey[5–7] and social interactions[8–10], as well as abiotic factors such as temperature[11–13] and oxygen level[14–16] are hypothesized or known to play important roles in shaping the evolution of developmental features[4,17], including developmental plasticity[18–24]. Direct tests for such ecological causality can be performed with experimental evo-devo, i.e. evolution experiments that examine hypotheses about multicellular development[25].

Many eco-evo-devo studies focus on direct selective relationships, i.e., on how a developmental process responds evolutionarily to selection by an ecological factor on that process. However, genetic causation of a phenotype that becomes important to fitness at some stage of a lineage's history might have first evolved in a hidden, non-adaptive manner. Many phenotypes are manifested in only a limited set of ecological contexts[26], which have been referred to as "inductive environments"[27,28]. The genetic basis of a focal phenotype might first evolve in a non-inductive environment (by any evolutionary mechanism, e.g. adaptation or genetic drift) while the actual phenotype is only first manifested, and thus revealed to potential selection, after later exposure to an inductive environment. Such evolution of initially latent phenotypes - latent-phenotype evolution (LPE[29,30], see *Semantics* in Methods) - is common[29–33] contributes to evolutionary diversification[29,30] and can fuel some forms of exaptation[26,34–36].

Studying the evolutionary history of developmental-phenotype causation is notoriously challenging in most natural systems, for which the relevant details of genetic, phenotypic and ecological history are often unknown. This is perhaps particularly true for organisms for which multicellular development is obligate, and for deciphering whether genetic causation of a phenotype initially evolved latently and thus non-adaptively. Ideal organisms for such studies would be both amenable to experimental evolution and characterized by a developmental process that is facultative and environment specific and which generates readily-quantifiable morphological phenotypes.

Microbes that undergo genetically-programmed multicellular development in response to specific environmental conditions are powerful model systems for experimental studies in eco-evo-devo[37]. Among prokaryotes, predatory myxobacteria are probably the most recognized in this regard, especially the model species *Myxococcus xanthus*[37], which is found in many soil habitats worldwide[38]. In the vegetative-growth phase of their multicellular life cycles, many

myxobacteria kill and consume diverse microbes as prey (both prokaryotic and eukaryotic) by molecular mechanisms that remain to be well-characterized[39,40]. *M. xanthus* employs two mechanistically distinct (but pleiotropically connected) modes of motility to migrate in search of prey and other resources and to carry out aggregative multicellular development. One system, traditionally known as 'S-motility,' is mediated by Type IV pili[41] and the other, traditionally known as 'A-motility,' is hypothesized to function by active transport of focal-adhesion protein complexes attached to the substratum[42]. The developmental phase of myxobacterial life cycles exhibited by many species is triggered by nutritional depletion, upon which cells aggregate to cooperatively construct elevated multicellular fruiting bodies. Some cells within fruiting bodies differentiate into spores capable of surviving harsh environmental conditions such as extended nutrient deprivation, high temperatures and likely predation[43–45].

Pleiotropic effects of adaptation - whether exerted across ecologically distinct growth environments[46–48] or across distinct life-history stages[37,49–54] - are, collectively, a central feature of evolution. Focusing on distinct life-history stages, degrees of pleiotropic connectedness among the genetic systems that underlie stage-specific traits are critical determinants of how life cycles can evolve[54,55]. Genetic and evolutionary connections between growth vs developmental phases of myxobacterial life cycles have received some attention[56–60], but require much further investigation. Adaptation to some prey environments fueling population growth may pleiotropically impact fruiting body formation during subsequent bouts of starvation. Indeed, adaptation of *M. xanthus* to predatory growth while foraging for *Escherichia coli* in a previous study resulted in a general decrease in fruiting-body numbers during starvation suggestive of a tradeoff between predatory fitness and development[57]. Trade-offs from systematically antagonistic pleiotropic effects of mutations adaptive in one life-history phase on performance at another phase[50,61,62] have major implications for life-cycle evolution as a whole and might promote some forms of diversity[62,63]. However, the occurrence and character of such life-cycle pleiotropy may often be specific to the ecological context in which the causal mutations evolve[62], a hypothesis we test here.

Fruiting body morphology has diversified strikingly among myxobacterial species (order Myxococcales)[38], as have many developmental traits already at the intra-specific level among *M. xanthus* lineages, including fruiting body traits[9,64,65], nutrient[66] and cell-density[67] thresholds triggering development, developmental timing[66] and even the genetic elements required for development[68]. However, the relative contributions of various forms of selection and stochastic forces to developmental diversification among myxobacteria remain unclear[37].

Forms of selection that may play major roles in diversifying fruiting body morphologies include i) direct selection by abiotic or biotic ecological factors on morphology *per se,* ii) selection on non-morphological aspects of development (e.g. the timing of cell-cell signaling)

that may nonetheless impact final fruiting body morphology and iii) selection on non-developmental life-cycle behaviors such as predation and growth-phase motility that pleiotropically impact development. Different biotic and abiotic factors such as nutrient level[69], substrate stiffness[70] and social environment[56,65,71] are known to affect *M. xanthus* developmental phenotypes, with likely implications for the evolution of fruiting body morphology[9]. The experimental inducibility and facultative character of *M. xanthus* multicellular development, and its versatility for experimental evolution, make this bacterium a powerful system for investigating the evolution of latent developmental phenotypes. Such phenotypes include the character and degree of phenotypic plasticity, which can be important for fitness and future evolution across variable environments[22,27,70,72].

We test the hypothesis that the ecological context of evolution by *M. xanthus* during growth can determine how such evolution latently alters developmental phenotypes. To do so, we use evolved populations from MyxoEE-3[73], an evolution experiment in which *M. xanthus* populations were selected for increased fitness at the leading edge of growing colonies that swarmed across agar-surface environments that differed at one or more ecological variables. Importantly, MyxoEE-3 populations were not experimentally subjected to starvation-induced development, such that evolutionary change in development-specific traits does not reflect adaptation for improved developmental fitness, but rather either pleiotropic effects of selection on other traits or stochastic evolution.

Here we analyzed two distinct sets of MyxoEE-3 treatments (Table S1)[74]. One set - Treatment Set A (TS-A) - included replicate populations that had evolved on either of two high-nutrient agar surfaces, one stiff surface with a high agar concentration (1.5%, CTT hard agar, or 'CTT-HA') and one soft surface with a low agar concentration (0.5%, CTT soft agar, or 'CTT-SA'). The TS-A treatments were analyzed after 40 cycles of MyxoEE-3 selection. On 0.5% agar, swarming of *M. xanthus* reference strains is driven almost exclusively by Type-IV-pili-mediated S-motility, whereas swarming on 1.5% agar is driven by a combination of A-motility and S-motility[57,73,75].

A second set of treatments - Treatment Set B (TS-B) - was composed of populations from five MyxoEE-3 treatments (Tables S1 and S2), including one shared with TS-A (CTT-HA) and four treatments in which *M. xanthus* was offered either *Bacillus subtilis* or *E. coli* bacterial species (Gram+ and Gram-, respectively) as prey. Among the four TS-B treatments with prey, two were identical to the CTT-HA treatment except that a lawn of either *B. subtilis* (*B. subtilis*-CTT) or *E. coli* (*E. coli*-CTT) was allowed to grow on the CTT hard-agar culture plates prior to their inoculation with *M. xanthus*. In this case, both the prey and any nutrients unused by the prey that remained in the agar substrate were available to *M. xanthus*. In the two other TS-B treatments (*B. subtilis*-TPM and *E. coli*-TPM), prey were first grown to stationary phase in

high-nutrient CTT liquid and then spread as high-density lawns onto buffered TPM agar lacking casitone, the carbon growth substrate present in CTT, before plates were inoculated with *M. xanthus*. In this case, nutrients were only available to *M. xanthus* from the prey. All TS-B treatments had plates with high (1.5%) agar concentration and were analyzed after 18 cycles of MyxoEE-3.

Among the evolved TS-A and TS-B populations of MyxoEE-3 examined here and their ancestors (Table S1, see Methods), we analyzed variation at four morphological traits associated with fruiting body formation as well as variation at spore production. We quantified the degree of phenotypic divergence from the ancestor and tested for effects of MyxoEE-3 selective environments on developmental LPE, including for treatment-level means for each trait, phenotypic plasticity for the TS-A treatments and degrees of stochastic diversification.

RESULTS

Substrate stiffness determines the character of developmental LPE

We quantitatively compared LPE of fruiting body (FB) morphology across MyxoEE-3 populations from both the TS-A and TS-B treatment sets and the ancestor (Anc). To do so, we used previously published methods of quantifying four morphological FB traits[9]: three trait medians measured at the resolution of individual FBs (density, density heterogeneity and area) and total FB number per assay plate (see Methods and La Fortezza and Velicer[9]). Considering TS-A first, microscopic observation (Fig. 1A), PCA of the entire morphological-trait data set (Fig. 1B) and individual-trait analysis (Fig. 1C, D) collectively reveal that both TS-A treatments diverged morphologically from the ancestor but clearly did so in a treatment-specific manner.

The PCA indicated treatment-level differentiation collectively across all traits (perMANOVA: $F = 42.9$, $R^2 = 0.94$, $Pr(>F) = 0.005$). In this analysis, PC1 explained the majority of the variance, with all eigenvectors making similar contributions to shaping the FB morphospace (Fig. S1A,B). More specifically, evolution on nutrient-rich soft agar (CTT-SA) indirectly led to greater FB morphological divergence from Anc than did evolution on hard agar (CTT-HA) (Fig. 1A). In further support of this observation, k-means and hierarchical-clustering analyses based on the degree of morphological similarity in the morphospace, also found the CTT-SA populations to be collectively distinct from both the CTT-HA populations and Anc, which together formed one statistical cluster (Fig. S1C-E).

Considering traits individually, average FB density and heterogeneity decreased on average from Anc among both CTT-HA and CTT-SA populations, whereas only CTT-SA populations evolved greatly from Anc in FB area and total counts, doing so by largely losing

the ability to form FBs at all (Fig. 1C, Fig. S1F). CTT-HA populations remained very similar to Anc in the latter two traits, thereby explaining their lower overall divergence from Anc across all traits. We then also examined LPE of sporulation efficiency and found trends comparable to those observed for the overall morphological analysis, in that CTT-HA populations retained levels of sporulation similar to Anc, whereas spore counts drastically decreased in CTT-SA populations (Fig. 1D, Fig. S1F). Under high-nutrient conditions, adaptation to swarming on a soft surface traded off against general developmental proficiency, whereas adaptation to swarming across a harder surface did not.

In light of the systematic latent loss of development in the CTT-SA lines, we examined previously reported mutation profiles of clones isolated from each of the CTT-HA and CTT-SA cycle-40 populations for possible candidate mutations[74] (one clone per population). In neither treatment were mutations preferentially localized among genes reported to be transcribed in a development-specific manner[74,76] (Fig. S2, Table S3, see Methods), as might be expected given the lack of selection on development during MyxoEE-3. However, an instance of gene-level parallel mutation specific to the CTT-SA treatment may represent the most common genetic route of developmental degradation among the CTT-SA populations. Among all loci mutated in more than one population (whether in CTT-HA or CTT-SA), the gene *lonD* was a hotspot of selection uniquely in the CTT-SA treatment, in which it was mutated in four populations (P29, P31, P33 and P35)[74], whereas it was not mutated in any CTT-HA population. *lonD* (aka *bsgA*) encodes an ATP-dependent protease that is required for developmental proficiency[77,78]. The histidine-kinase gene *MXAN_5852* (*MXAN_RS28370*) was also mutated in parallel in the CTT-SA treatment (four populations). However, previous experimental mutation of this gene did not cause major decreases in spore production[79], suggesting that the MyxoEE-3 mutations found in this gene may not be responsible for the losses of developmental proficiency observed in the respective populations. We thus speculate that mutation of *lonD* is likely to have been a shared route of developmental loss among four CTT-SA populations, while at least one other route was taken by the other two populations examined here.

While all six TS-A CTT-HA populations retained relatively high sporulation levels compared to the CTT-SA populations, three nonetheless evolved partial reductions of spore production (Table S3). The sequenced clones from those three populations each had a mutation in the histidine-kinase gene *hsfB*, which was not mutated in the other three populations that retained full ancestral proficiency at development. HsfB has been shown to phosphorylate the response regulator HsfA, which in turn activates transcription of l*onD*[80]. Thus, gene members of the same *hsfB*/*hsfA*/*lonD* developmental regulatory pathway were

clearly targeted by selection in both the CTT-HA and CTT-SA treatments, but the specific gene targeted differed between treatments.

Latent evolution of developmental plasticity

Beyond phenotypes manifested in a single environment, the degree and character of developmental plasticity across environmental gradients might also evolve latently and thus at least initially remain hidden to selection[81,82]. *M. xanthus* developmental phenotypes often vary as a function of agar concentration[70]. *M. xanthus* developmental phenotypes often vary as a function of agar concentration50. This ancestral phenotypic plasticity allowed us to ask whether developmental-phenotype reaction norms of TS-A-treatment populations across an agar-concentration gradient (0.5%, 1.0% and 1.5%) evolved latently and, if so, whether the MyxoEE-3 selective environment impacted the character of such latent reaction-norm evolution (Fig. S3A,B).

The reaction norms of both CTT-HA and CTT-SA populations evolved latently at the treatment level (Fig. 2A and Fig. S3C-E), but in very different manners. In the CTT-HA treatment, average reaction norms were altered significantly relative to the Anc clones in overall mean, linear slope and/or shape for three of the four examined traits (Fig. 2A). Intriguingly, however, considering average reaction norms at the treatment level masks extensive stochastic diversification of reaction norms across individual populations at all four morphological traits (Fig. 2B). Indeed, for all four traits, some populations varied not only in slope, but in the very sign of the trait-value vs. agar-concentration relationship, whether across all three agar concentrations or only two (Fig. 2B). Thus, for this treatment, although reaction norms did evolve mildly at the treatment level (Fig. 2A), stochastic variation in mutational input between replicate populations (the only plausible explanation for phenotypic variation between population replicates) mattered much more for reaction-norm evolution than did the selective conditions of the CTT-HA environment of MyxoEE-3. Intriguingly, this stochastic radiation led to repeated examples of increased plasticity (e.g. P3 and P11 for FB counts), phenotypic canalization (reaction-norm slope reduction, e.g. P9 and all CTT-SA populations for FB counts), qualitatively novel reaction-norm patterns (e.g. P9 for FB counts) and evolutionary reversals of reaction-norm slopes (e.g. P5 and P9 for FB area) (Fig. 2B).

In contrast to the CTT-HA treatment, all six of the CTT-SA populations evolved in parallel to become completely or largely non-plastic. This is because the previously noted inability of these populations to form fruiting bodies on 1.5% agar (Fig. 1C) was also observed at the other two agar concentrations (Fig. 2A and Fig. S3B). Also, unlike CTT-HA, which allowed much stochastic latent diversification of reaction norms, CTT-SA greatly constrained reaction-

norm LPE to yield nearly identically flat evolved reaction norms all across populations (Fig. 2B).

Prey presence and identity also strongly shape developmental LPE

Myxobacterial adaptation to distinct prey environments might drive diversification at many traits, including predatory performance and mechanisms across diverse prey types[83], social competitiveness[60,84], forms and degrees of cooperation during predation[85], motility performance[29,57], secondary-metabolite production*, production of or sensitivity to antibiotics and, a hypothesis we test here, development-related phenotypes. Overall, PCA of the entire morphological datasets run on all five treatments in TS-B, four of which included prey, showed some form of clear divergence of fruiting body morphology relative to Anc (Fig. 3A-B, (perMANOVA: F = 50.5, R2 = 0.95, Pr(>F) = 0.001), Fig. S4A-B). Moreover, most pairwise treatment comparisons reveal treatment-level diversification at one or more developmental traits (Fig. 3C, Fig. S4F). Most broadly, the presence of prey, irrespective of abiotic context, tended to promote greater evolutionary change, as the four treatments that included prey bacteria all clearly or apparently diverged more from Anc than did the cycle-18 CTT-HA populations (Fig. 3B, 3C), which, like the cycle-40 CTT-HA populations examined in TS-A (Fig. 1B, 1C), remained relatively similar to Anc.

Three TS-B treatments shared the same abiotic environment of high-nutrient CTT hard agar, differing only in the presence or absence of any prey or in prey identity - *B. subtilis* or *E. coli* (CTT-HA, *B. subtilis*-CTT, *E. coli*-CTT). Among these three abiotically identical treatments, the presence of either prey species resulted in greater morphological evolution than did the absence of prey (Fig. 1C). However, the two prey species had different effects on LPE, with greater indirect morphological evolution occurring in the *B. subtilis*-CTT treatment than in the *E. coli*-CTT treatment (Fig. 3B, 3C).

Consistent with this result, *B. subtilis* also caused greater LPE than *E. coli* between the *B. subtilis*-TPM vs *E. coli*-TPM treatments (Fig. 3B, 3C). Indeed, prey identity mattered even more for LPE than the differences between the CTT vs TPM treatments with prey (Table S2), in that both *B. subtilis* treatments showed greater divergence from Anc and from the CTT-HA treatment than either *E. coli* treatment (Fig. 3B, 3C). Highlighting this primary effect of prey identity, k-means cluster analysis of all five TS-B treatments identified two primary statistical clusters distinguished solely by the presence or absence of *B. subtilis* (Fig. S4D). Consistent with the above collective analyses (PCA and k-means), both *B. subtilis* treatments exhibited significantly reduced trait values for all four individually examined morphological traits, whereas the *E. coli* treatments each diverged from Anc only at a subset of traits (Fig. 3C).

As for TS-A, we examined levels of sporulation from all TS-B evolved populations. Also in this case, MyxoEE-3 selective environments strongly determined LPE of sporulation. In detail, sporulation level decreased greatly relative to Anc for those populations evolved in the presence of *B. subtilis* in either abiotic context and in the *E. coli*-CTT populations, whereas it remained near the ancestral level for both CTT-HA and *E. coli*-TPM evolved populations (Fig. 3D).

In Myxococcus literature, sporulation is frequently, if not always, associated positively with FB formation. While the two developmental processes are indeed often linked, we[37,86] have recently shown that sporulation can evolutionarily become decoupled from fruiting-body development, as occurred during MyxoEE-7[9]. We observed that MyxoEE-3 treatments that evolved large reductions in morphological-trait values also showed large decreases in sporulation (Fig. 3C, 3D) and therefore formally tested whether sporulation and FB counts correlate positively after MyxoEE-3 evolution (for all TS-A and TS-B treatments pooled together). Indeed, reductions in FB counts were quantitatively associated with reduced spore counts ($r = 0.88$, $p = 4.4 \times 10^{-15}$) (Fig. S5).

Taken together, our results indicate that both abiotic and biotic details of selective environments during myxobacterial growth can greatly impact the evolution of latent developmental phenotypes that are revealed only upon exposure to starvation. Specifically, agar concentration in the physical substrate on which *M. xanthus* evolved and the identity of prey consumed by *M. xanthus* both had large indirect effects on the evolutionary fate of fruiting body morphology and sporulation efficiency.

Distinct selective environments differentially limit stochastic diversification of fruiting body morphology

Replicate experimental populations evolving under the same selective pressures often diversify stochastically[87,88]. However, the character of the selective environment can limit the degree of such stochastic diversification, whether for immediately manifested or latent phenotypes[9,29,30,89]. For example, we have recently shown that social selection acting during *M. xanthus* development can limit the degree of stochastic morphological diversification among evolving populations[9]. Here we asked whether such limitation of stochastic developmental diversification can also occur when selection operates solely during *M. xanthus* population growth rather than on starvation-induced development. To do so, we quantified morphological dispersion across all replicate populations within each individual treatment of both TS-A and TS-B from the previously obtained morphospaces (Fig. 1B and 3B) (see Methods).

We find that both agar concentration in TS-A and the presence of prey (of either identity) in TS-B determined the degree of stochastic evolutionary diversification at developmental phenotypes during MyxoEE-3. In TS-A, replicate populations evolved on CTT-HA diversified much more than the CTT-SA populations (Fig. 4A, Fig. S6A,B), a pattern corroborated by the average variances of individual-trait values across populations (Fig. 4B). FB counts and FB area explained most of the inter-population diversification observed for CTT-HA in TS-A (Fig. 4B, Fig. S1F). Evolution on CTT-SA resulted in extremely low levels of inter-population diversity at the end of MyxoEE-3, which was not significantly greater than diversity among the Anc founding clones (Fig. 4A, Fig. S6A,B), despite the extensive collective morphological divergence of the CTT-SA treatment away from its ancestral state (Fig. 1B, 1C and Fig. S1D, E). This lack of diversification is due to the systematic parallel loss of developmental proficiency by all six populations examined in this treatment (Fig. 1A, B, D and Fig. S1F).

Stochastic morphological diversification was also indirectly limited by specific TS-B selective environments. Evolution in the presence of either prey species on CTT hard agar allowed greater latent diversification of developmental phenotypes than evolution in the absence of prey, both across the collective morphospace (Fig. 4C, Fig. S6C,D) and often at single traits considered individually (Fig. 4D, Fig. S4F), as did adaptation to *E. coli* as prey on TPM agar. Stochastic diversification was lowest on *B. subtilis*-TPM, largely due to systematic parallel reductions of trait values across the four populations (Fig. 3A, C, D and Fig. S4F) (as occurred also in the CTT-SA populations of TS-A (Fig. 1A, B, D and Fig. S1F).

Comparing treatments sharing the same prey species (*B. subtilis*-CTT vs *B. subtilis*-TPM; *E. coli*-CTT vs *E. coli*-TPM), *M. xanthus* diversification was lower in the TPM treatments than the CTT treatments (Fig. 4C). Collectively, these results demonstrate that stochastic developmental diversification mediated by LPE can be strongly influenced by both abiotic and biotic components of the selective environment, here surface stiffness and prey environment, respectively.

DISCUSSION

Phenotype expression and form often depend greatly on ecological context. Genetic causation of phenotypic novelty can thus evolve latently (hidden from selection) in one environment, until the causal genotype is exposed to a different, inductive environment that triggers phenotypic manifestation. Here we have shown how both abiotic and biotic ecological factors - swarming-surface stiffness and predator-prey interactions, respectively - shape and limit the evolution of latent developmental phenotypes in the multicellular bacterium *M. xanthus*.

Morphological diversification

The sophisticated genetic and behavioral complexity of fruiting body development among myxobacteria clearly points to these forms of aggregative multicellularity conferring significant evolutionary benefits. But what those benefits are remains unclear and may include protection from abiotic stresses and biotic dangers (e.g. potential predation by nematodes[90]), enhanced dispersal, and positive effects of high density during spore germination and post-germination growth and predation[37]. Even less clear is any adaptive significance to the striking diversification of fruiting body morphology observed across myxobacterial species (or similarly across aggregative developmental species of dictyostelids)[37,38]. One evolution experiment - MyxoEE-7 - has identified selective forces acting on fitness during *M. xanthus* development that can drive morphological diversification, namely selection mediated by distinct social environments[9].

Our results with MyxoEE-3 suggest that evolutionary processes other than selection on fitness during development are also likely to play major roles in the morphological diversification of aggregative developmental systems. Ecological differences during growth without development - including a simple physical difference in growth surface and a simple difference in the identity of one-species prey environments - are found to evolutionarily shape not only details of fruiting body morphology in environments that do induce development, but the very evolutionary persistence of development itself (Figs. 1A-C and Fig. 3A-C). Further investigations of how evolutionary forces other than selection on development per se interact with such selection to impact fruiting body evolution, including increases in morphological complexity, will be of interest.

Pleiotropy and life-cycle evolution

The relative origins and long-term evolutionary integration of the genetic systems enabling the motility, predation, aggregative development and germination components of myxobacterial multicellular life cycles remain to be thoroughly characterized[91,92]. However, the pervasiveness of LPE emergent from MyxoEE evolution experiments[29,30,56,57,59,60,74,84,86,93] and of pleiotropy across *M. xanthus* behaviors from mutations engineered or induced in mechanistic molecular studies[58,94] together indicate that many loci contribute to more than one of these behaviors. Increasingly systematic and extensive investigations of pleiotropy across *M. xanthus* behaviors under standardized conditions will provide greater insight into the shared vs. modular components of the genetic systems underlying these behaviors and their evolution[55].

When selection operates across whole myxobacterial life cycles, trade-offs mediated by antagonistic pleiotropy that may exist between adaptive improvement at any one life-history

stage (e.g. population growth fueled by predation) and another (e.g. development) must be balanced[54]. Whether such trade-offs even exist and the detailed manner in which selection balances those that do may often depend on details of the ecological context within which those life cycles are played out. Suggesting that this will often be the case in natural contexts, MyxoEE-3 reveals profound differences in the character of developmental LPE as a function of the ecological details of vegetative growth, for example the presence vs absence of prey and the identity of prey.

That adaptation to distinct prey environments when selection on development is relaxed often has large divergent effects on development suggests that prey environments will also impact developmental-system divergence when selection across whole life cycles favors high proficiency at both predation and development. To address this question, evolution experiments might be performed that include both predatory growth and starvation-induced development within each selection cycle and multiple treatments that differ only in prey environment (perhaps with respect to both prey identity and prey-community complexity). Distinct prey environments may drive divergence at nutritional triggers of development, developmental timing, overall patterns of developmental gene expression, sporulation level, mechanisms of cheater-cooperator coevolution (for developmental cheating as well as cheating on cooperative predation traits), the relationship between fruiting-body formation and sporulation, fruiting-body morphology, and the molecular triggers and social character of spore germination to reinitiate predatory growth, and might even ultimately drive diversification of the very gene sets necessary for development[76,92]. Other experiments might be performed to test how, reciprocally, selection on different developmental traits (e.g. developmental timing or spore quality) might differentially impact predatory performance across various prey environments.

In the first evolution experiment with *M. xanthus* (MyxoEE-1), most replicate populations adapting to growth in nutrient-rich CTT liquid latently evolved large decreases in developmental proficiency, including at both fruiting-body formation and sporulation[86]. However, the relative contributions of nutrient abundance vs absence of a solid growth substratum, if any, to promoting the evolutionary degradation of development in MyxoEE-1 have been unclear. Our analyses of MyxoEE-3 suggest that mere relaxation of selection for developmental proficiency by provision of abundant growth resources is often less important to the evolutionary fate of development than other details of the ecological context in which population growth occurs. Development was largely retained when growth under nutrient abundance was accompanied by swarming that employs both *M. xanthus* motility systems (on hard agar), whereas it tended to be lost when S-motility alone (on soft agar) (Fig. 3) or no motility at all (Velicer et al. 1998) was employed during growth (Table S2). Developmental

phenotypes were retained at much higher levels when *E. coli* was consumed as prey than when *B. subtilis* was consumed, regardless of abiotic context (Fig. 3).

Comparing only the TS-A treatments, it appears that a mere difference in the relative employment of the two *M. xanthus* motility systems during growth determines the evolutionary fate of development. Evolution while swarming almost exclusively by S-motility on soft agar (at least in the ancestral state) led to systematic severe losses of developmental proficiency. In contrast, swarming on a hard-agar surface while more equally employing both A-motility and S-motility[75] led to only sporadic and relatively mild decreases in developmental phenotypes (Fig. 1D, Fig. S4F) Intriguingly, the simple difference in surface stiffness between the CTT-HA vs CTT-SA regimes also led to selection on different genes within the same regulatory pathway controlling multicellular development (*lonD*/*bsgA*). Future analysis of the precise effects of mutations in these genes (and other genes mutated in parallel during MyxoEE-3l) on fitness during swarming and on developmental phenotypes would likely provide novel insights into pleiotropic connections between development and the distinct mechanisms of A- and S- motility employed during vegetative growth.

In light of the TS-A results, the different LPE effects of distinct prey environments among the TS-B treatments might be due to differential effects of those prey environments on the relative employment of the two *M. xanthus* motility systems. Alternatively, differences in predator-prey interactions unrelated to motility may be at play.

Deterministic vs stochastic diversification of latent phenotypes

Our experiments reveal both deterministic and stochastic forms of latent-phenotype diversification (LPD). In deterministic LPD, populations diversified systematically at the treatment level due to differences in the average pleiotropic effects on development of adaptive mutations that arose in different growth environments. In stochastic LPD, replicate populations within the same treatment diverged from one another over time, an outcome explained by stochastic variation across replicate evolving populations in the identity and/or temporal order of mutations that occurred within each. While such stochastic LPD is evident already from examining individual traits in single environments (Fig. S8), its perhaps most striking manifestation in our data set is the remarkable diversification of developmental reaction norms among the CTT-HA populations (Fig. 2B, Fig. S2). Among these populations, the very sign of reaction-norm slopes diversified stochastically for all four examined morphological traits across part or all of the examined environmental gradient (Fig. 2B). Phenotypic plasticity is well recognized as often being important for fitness, evolutionary trajectories and evolvability across variable environmental conditions[95]. Latently-evolved

reaction-norm diversity might fuel novel routes of adaptive innovation during future evolution through changing environments[81,82].

The likely contributions of LPE to long-term evolutionary processes have been receiving increasing attention[29–33,36]. The outcomes of MyxoEE-3 reported here suggest that not only LPE per se, but the ecological context in which LPE originates, may often be important for the evolution of many developmental systems.

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Data availability. All **datasets** are available at figShare (see figure legends). Raw images and programming codes used for the analyses are available upon request to the corresponding author.

Competing interest statement. The authors do not declare any conflict of interest

REFERENCES

1. Carroll, S.B. (2005). Endless Forms Most Beautiful: The New Science of Evo Devo and the Making of the Animal Kingdom. (New York, U.S.A.: W. W. Norton eds.)

2. Dobzhansky, T., and Spassky, B. (1944). Genetics of natural populations. XI. manifestation of genetic variants in *Drosophila pseudoobscura* in different environments. Genetics ***29***, 270–90.

3. Abouheif, E., Fave, M.-J., Ibarraran-Viniegra, A.S., Lesoway, M.P., Rafiqi, A.M., and Rajakumar, R. (2014). Eco-Evo-Devo: The Time Has Come. In Ecological Genomics. Advances in Experimental Medicine and Biology, vol 781. (Springer, Dordrecht). 10.1007/978-94-007-7347-9_6

4. Sultan, S.E. (2021). Eco-Evo-Devo. In Evolutionary Developmental Biology. (Springer, Cham.) 10.1007/978-3-319-32979-6_42

5. Gomez-Mestre, I., and Warkentin, K.M. (2007). To hatch and hatch not: similar selective trade-offs but different responses to egg predators in two closely related, syntopic treefrogs. Oecologia ***153***, 197–206. 10.1007/s00442-007-0708-0

6. Herron, M.D., Borin, J.M., Boswell, J.C., Walker, J., Chen, I.-C.K., Knox, C.A., Boyd, M., Rosenzweig, F., and Ratcliff, W.C. (2019). De novo origins of multicellularity in response to predation. Sci Rep ***9***, 2328. 10.1038/s41598-019-39558-8

7. Fisher, R.M., Bell, T., and West, S.A. (2016). Multicellular group formation in response to predators in the alga *Chlorella vulgaris*. J Evol Biol ***29***, 551–559. 10.1111/jeb.12804

8. Noh, S., Christopher, L., Strassmann, J.E., and Queller, D.C. (2020). Wild *Dictyostelium discoideum* social amoebae show plastic responses to the presence of nonrelatives during multicellular development. Ecol Evol ***10***, 1119–1134. 10.1002/ece3.5924

9. La Fortezza, M., and Velicer, G.J. (2021). Social selection within aggregative multicellular development drives morphological evolution. bioRxiv. 10.1101/2021.05.12.443771

10. West-Eberhard, M.J. (1983). Sexual selection, social competition, and speciation. Q Rev Biology ***58***, 155–183. 10.1086/413215

11. Saxon, A.D., O'Brien, E.K., and Bridle, J.R. (2018). Temperature fluctuations during development reduce male fitness and may limit adaptive potential in tropical rainforest Drosophila. J Evol Biol ***31***, 405–415. 10.1111/jeb.13231

12. García-Roa, R., Garcia-Gonzalez, F., Noble, D.W.A., and Carazo, P. (2020). Temperature as a modulator of sexual selection. Biol Rev ***95***, 1607–1629. 10.1111/brv.12632

13. McGlashan, J.K., Spencer, R.-J., and Old, J.M. (2011). Embryonic communication in the nest: metabolic responses of reptilian embryos to developmental rates of siblings. Proc. R. Soc. B. ***279***, 1709–15. 10.1098/rspb.2011.2074

14. Bozdag, G.O., Libby, E., Pineau, R., Reinhard, C.T., and Ratcliff, W.C. (2021). Oxygen suppression of macroscopic multicellularity. Nat Commun ***12***, 2838. 10.1038/s41467-021-23104-0

15. Reinhard, C.T., Planavsky, N.J., Olson, S.L., Lyons, T.W., and Erwin, D.H. (2016). Earth's oxygen cycle and the evolution of animal life. Proceedings of the National Academy of Sciences of the U.S.A. ***113***, 8933–8938. 10.1073/pnas.1521544113

16. Callier, V., and Nijhout, H.F. (2011). Control of body size by oxygen supply reveals size-dependent and size-independent mechanisms of molting and metamorphosis. Proceedings of the National Academy of Sciences of the U.S.A. ***108***, 14664–14669. 10.1073/pnas.1106556108

17. Taubenheim, J., Willoweit-Ohl, D., Knop, M., Franzenburg, S., He, J., Bosch, T.C.G., and Fraune, S. (2020). Bacteria- and temperature-regulated peptides modulate β-catenin signaling in Hydra. Proceedings of the National Academy of Sciences of the U.S.A., 202010945. 10.1073/pnas.2010945117

18. Sikkink, K.L., Reynolds, R.M., Ituarte, C.M., Cresko, W.A., and Phillips, P.C. (2014). Rapid evolution of phenotypic plasticity and shifting thresholds of genetic assimilation in the nematode *Caenorhabditis remanei*. G3 ***4***, 1103–12. 10.1534/g3.114.010553

19. Scoville, A.G., and Pfrender, M.E. (2010). Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. Proceedings of the National Academy of Sciences of the U.S.A. ***107***, 4260–4263. 10.1073/pnas.0912748107

20. Nijhout, H.F., Kudla, A.M., and Hazelwood, C.C. (2020). Genetic assimilation and accommodation: Models and mechanisms. In Current Topics in Developmental Biology, pp. 337–369. (Academic Press). 10.1016/bs.ctdb.2020.11.006

21. Hintze, M., Koneru, S.L., Gilbert, S.P.R., Katsanos, D., Lambert, J., and Barkoulas, M. (2020). A cell fate switch in the *Caenorhabditis elegans* seam cell lineage occurs through modulation of the Wnt asymmetry pathway in response to temperature increase. Genetics ***214***, 927–939. 10.1534/genetics.119.302896

553 22. Paaby, A.B., and Testa, N.D. (2021). Developmental Plasticity and Evolution. In
554 Evolutionary Developmental Biology. (Springer, Cham.) 10.1007/978-3-319-32979-6_110

555 23. Sultan, S.E. (n.d.). Phenotypic Plasticity as an Intrinsic Property of Organisms. In
556 Phenotypic Plasticity & Evolution. (CRC Press)

557 24. Gonzalez, P.N., and Barbeito-Andrés, J. (2018). Canalization: A Central but Controversial
558 Concept in Evo-Devo. In Evolutionary Developmental Biology. (Springer, Cham.)
559 10.1007/978-3-319-33038-9_55-1

560 25. Kawecki, T.J., Lenski, R.E., Ebert, D., Hollis, B., Olivieri, I., and Whitlock, M.C. (2012).
561 Experimental evolution. Trends Ecol Evol ***27***, 547–560. 10.1016/j.tree.2012.06.001

562 26. Wagner, A. (2017). The white-knight hypothesis, or does the environment limit
563 innovations? Trends Ecol Evol ***32***, 131–140. 10.1016/j.tree.2016.10.017

564 27. Lafuente, E., and Beldade, P. (2019). Genomics of developmental plasticity in animals.
565 Front Genet ***10***, 720. 10.3389/fgene.2019.00720

566 28. Nijhout, H.F. (1990). Problems and paradigms: metaphors and the role of genes in
567 development. Bioessays ***12***, 441–446. 10.1002/bies.950120908

568 29. Rendueles, O., and Velicer, G.J. (2020). Hidden paths to endless forms most wonderful:
569 Complexity of bacterial motility shapes diversification of latent phenotypes. BMC Evol Biol
570 ***20***, 145. 10.1002/bies.950120908

571 30. Freund, L., Vasse, M., and Velicer, G.J. (2021). Hidden paths to endless forms most
572 wonderful: parasite-blind diversification of host quality. Proc R Soc B ***288***, 20210456.
573 10.1098/rspb.2021.0456

574 31. Kinsler, G., Geiler-Samerotte, K., and Petrov, D.A. (2020). Fitness variation across subtle
575 environmental perturbations reveals local modularity and global pleiotropy of adaptation.
576 eLife ***9***, e61271. 10.7554/elife.61271

577 32. Paaby, A.B., and Rockman, M.V. (2013). The many faces of pleiotropy. Trends Genet ***29***,
578 66–73. 10.1016/j.tig.2012.10.010

579 33. Paaby, A.B., and Rockman, M.V. (2014). Cryptic genetic variation: evolution's hidden
580 substrate. Nat Rev Genet ***15***, 247–258. 10.1038/nrg3688

581 34. Payne, J.L., and Wagner, A. (2014). Latent phenotypes pervade gene regulatory circuits.
582 BMC Syst Biol ***8***, 64. 10.1186/1752-0509-8-64

583 35. Payne, J.L., and Wagner, A. (2019). The causes of evolvability and their evolution. Nat
584 Rev Genet ***20***, 24–38. 10.1038/s41576-018-0069-z

585 36. Zheng, J., Payne, J.L., and Wagner, A. (2019). Cryptic genetic variation accelerates
586 evolution by opening access to diverse adaptive peaks. Science ***365***, 347–353.
587 10.1126/science.aax1837

588 37. La Fortezza, M., Schaal, K., and Velicer, G.J. (2021). Why Aggregate? On the Evolution
589 of Aggregative Multicellularity. Preprints. 10.20944/preprints202105.0451.v1

590 38. Dawid, W. (2000). Biology and global distribution of myxobacteria in soils. FEMS Microbiol
591 Rev ***24***, 403–427. 10.1111/j.1574-6976.2000.tb00548.x

592 39. Thiery, S., and Kaimer, C. (2020). The Predation Strategy of *Myxococcus xanthus*. Front
593 Microbiol ***11***, 2. 10.3389/fmicb.2020.00002

594 40. Arend, K.I., Schmidt, J.J., Bentler, T., Lüchtefeld, C., Eggerichs, D., Hexamer, H.M., and
595 Kaimer, C. (2020). *Myxococcus xanthus* predation of Gram-positive or Gram-negative
596 bacteria is mediated by different bacteriolytic mechanisms. Appl Environ Microb ***87***.
597 10.1128/aem.02382-20

598 41. Wu, S.S., and Kaiser, D. (1995). Genetic and functional evidence that Type IV pili are
599 required for social gliding motility in *Myxococcus xanthus*. Mol Microbiol ***18***, 547–558.
600 10.1111/j.1365-2958.1995.mmi_18030547.x

601 42. Faure, L.M., Fiche, J.-B., Espinosa, L., Ducret, A., Anantharaman, V., Luciano, J.,
602 Lhospice, S., Islam, S.T., Tréguier, J., Sotes, M., et al. (2016). The mechanism of force
603 transmission at bacterial focal adhesion complexes. Nature ***539***, 530–535.
604 10.1038/nature20121

605 43. Shimkets, L.J., Dworkin, M., and Reichenbach, H. (2006). The Myxobacteria. In The
606 Prokaryotes, Volume 7: Proteobacteria: Delta, Epsilon Subclass. pp 31–115. (Springer,
607 New York, NY). 10.1007/0-387-30747-8_3

608 44. Mercier, R., and Mignot, T. (2016). Regulations governing the multicellular lifestyle of
609 *Myxococcus xanthus*. Curr Opin Microbiol ***34***, 104–110. 10.1016/j.mib.2016.08.009

610 45. Muñoz-Dorado, J., Marcos-Torres, F.J., García-Bravo, E., Moraleda-Muñoz, A., and
611 Pérez, J. (2016). Myxobacteria: Moving, killing, feeding, and surviving together. Front
612 Microbiol ***7***, 2475–18. 10.3389/fmicb.2016.00781

613 46. Kawecki, T.J., and Ebert, D. (2004). Conceptual issues in local adaptation. Ecol Lett ***7***,
614 1225–1241. 10.1111/j.1461-0248.2004.00684.x

615 47. Levins, R. (1968). Evolution in Changing Environments, Some Theoretical Explorations.
616 (Princeton University Press).

617 48. Satterwhite, R.S., and Cooper, T.F. (2015). Constraints on adaptation of *Escherichia coli*
618 to mixed-resource environments increase over time. Evolution ***69***, 2067–2078.
619 10.1111/evo.12710

620 49. Williams, G.C. (1957). Pleiotropy, natural selection, and the evolution of senescence.
621 Evolution ***11***, 398. 10.2307/2406060

622 50. Ackermann, M., Schauerte, A., Stearns, S.C., and Jenal, U. (2007). Experimental evolution
623 of aging in a bacterium. BMC Evol Biol ***7***, 126. 10.1186/1471-2148-7-126

624 51. Moreno-Gámez, S., Kiviet, D.J., Vulin, C., Schlegel, S., Schlegel, K., Doorn, G.S. van, and
625 Ackermann, M. (2020). Wide lag time distributions break a trade-off between reproduction
626 and survival in bacteria. Proceedings of the National Academy of Sciences of the U.S.A.
627 ***117***, 18729–18736. 10.1073/pnas.2003331117

628 52. Paepe, M.D., and Taddei, F. (2006). Viruses' life history: Towards a mechanistic basis of
629 a trade-off between survival and reproduction among phages. PloS Biol ***4***, e193.
630 10.1371/journal.pbio.0040193

631 53. Zakrzewska, A., Eikenhorst, G. van, Burggraaff, J.E.C., Vis, D.J., Hoefsloot, H., Delneri,
632 D., Oliver, S.G., Brul, S., and Smits, G.J. (2011). Genome-wide analysis of yeast stress
633 survival and tolerance acquisition to analyze the central trade-off between growth rate and
634 cellular robustness. Mol Biol Cell ***22***, 4435–4446. 10.1091/mbc.e10-08-0721

635 54. Stearns, S.C. (1992). The evolution of life histories (Oxford University Press).

636 55. Wagner, G.P., and Zhang, J. (2011). The pleiotropic structure of the genotype–phenotype
637 map: the evolvability of complex organisms. Nat Rev Gen ***12***, 204–213. 10.1038/nrg2949

638 56. Velicer, G.J., Kroos, L., and Lenski, R.E. (2000). Developmental cheating in the social
639 bacterium *Myxococcus xanthus*. Nature ***404***, 598–601. 10.1038/35007066

640 57. Hillesland, K.L., Velicer, G.J., and Lenski, R.E. (2009). Experimental evolution of a
641 microbial predator's ability to find prey. Proc R Soc B ***276***, 459–467.
642 10.1098/rspb.2008.1098

643 58. Pham, V.D., Shebelut, C.W., Diodati, M.E., Bull, C.T., and Singer, M. (2005). Mutations
644 affecting predation ability of the soil bacterium *Myxococcus xanthus*. Microbiology ***151***,
645 1865–1874. 10.1099/mic.0.27824-0

646 59. Zee, P.C., Liu, J., and Velicer, G.J. (2016). Pervasive, yet idiosyncratic, epistatic pleiotropy
647 during adaptation in a behaviourally complex microbe. J Evol Biol ***30***, 257–269.
648 10.1111/jeb.12999

649 60. Nair, R.R., Fiegna, F., and Velicer, G.J. (2018). Indirect evolution of social fitness
650 inequalities and facultative social exploitation. Proc R Soc B ***285***, 20180054.
651 10.1098/rspb.2018.0054

652 61. Chen, P., and Zhang, J. (2020). Antagonistic pleiotropy conceals molecular adaptations in
653 changing environments. Nat Ecol Evol ***4***, 461–469. 10.1038/s41559-020-1107-8

654 62. Bono, L.M., Smith, L.B., Pfennig, D.W., and Burch, C.L. (2017). The emergence of
655 performance trade-offs during local adaptation: insights from experimental evolution. Mol
656 Ecol ***26***, 1720–1733. 10.1111/mec.13979

657 63. Ferenci, T. (2016). Trade-off mechanisms shaping the diversity of bacteria. Trends
658 Microbiol ***24***, 209–223. 10.1016/j.tim.2015.11.009

659 64. Amherd, M., Velicer, G.J., and Rendueles, O. (2018). Spontaneous nongenetic variation
660 of group size creates cheater-free groups of social microbes. Behav Ecol ***29***, 393–403.
661 10.1093/beheco/arx184

662 65. Fiegna, F., and Velicer, G.J. (2005). Exploitative and hierarchical antagonism in a
663 cooperative bacterium. PLoS Biology ***3***, e370. 10.1371/journal.pbio.0030370

664 66. Kraemer, S.A., Toups, M.A., and Velicer, G.J. (2010). Natural variation in developmental
665 life-history traits of the bacterium *Myxococcus xanthus*. FEMS Microbiol Ecol ***73***, 226-233.
666 10.1111/j.1574-6941.2010.00888.x

667 67. Kadam, S.V., and Velicer, G.J. (2006). Variable patterns of density-dependent survival in
668 social bacteria. Behav Ecol ***17***, 833–838. 10.1093/beheco/arl018

669 68. Rajagopalan, R., Wielgoss, S., Lippert, G., Velicer, G.J., and Kroos, L. (2015). *devI* is an
670 evolutionarily young negative regulator of *Myxococcus xanthus* development. Journal of
671 Bacteriology ***197***, 1249–1262. 10.1128/jb.02542-14

672 69. Harris, B.Z., Kaiser, D., and Singer, M. (1998). The guanosine nucleotide (p)ppGpp
673 initiates development and A-factor production in *Myxococcus xanthus*. Gene Dev ***12***,
674 1022–1035. 10.1101/gad.12.7.1022

675 70. Rivera-Yoshida, N., Arzola, A.V., Angel, J.A.A.D., Franci, A., Travisano, M., Escalante,
676 A.E., and Benítez, M. (2019). Plastic multicellular development of *Myxococcus xanthus*:
677 genotype–environment interactions in a physical gradient. R Soc open science ***6***, 181730–
678 9. 10.1098/rsos.181730

679 71. Fiegna, F., Yu, Y.-T.N., Kadam, S.V., and Velicer, G.J. (2006). Evolution of an obligate
680 social cheater to a superior cooperator. Nature ***441***, 310–314. 10.1038/nature04677

681 72. Levis, N.A., and Pfennig, D.W. (2019). Phenotypic plasticity, canalization, and the origins
682 of novelty: Evidence and mechanisms from amphibians. Seminars in Cell and
683 Developmental Biology ***88***, 80–90. 10.1016/j.semcdb.2018.01.012

684 73. Velicer, G.J., and Yu, Y.N. (2003). Evolution of novel cooperative swarming in the
685 bacterium *Myxococcus xanthus*. Nature ***425***, 75–78. 10.1038/nature01908

686 74. Rendueles, O., and Velicer, G.J. (2016). Evolution by flight and fight: diverse mechanisms
687 of adaptation by actively motile microbes. ISME J ***11***, 555–568. 10.1038/ismej.2016.115

688 75. Shi, W., and Zusman, D.R. (1993). The two motility systems of *Myxococcus xanthus* show
689 different selective advantages on various surfaces. Proceedings of the National Academy
690 of Sciences of the U.S.A. ***90***, 3378–3382. 10.1073/pnas.90.8.3378

691 76. Muñoz-Dorado, J., Moraleda-Muñoz, A., Marcos-Torres, F.J., Contreras-Moreno, F.J.,
692 Martin-Cuadrado, A.B., Schrader, J.M., Higgs, P.I., and Pérez, J. (2019). Transcriptome
693 dynamics of the *Myxococcus xanthus* multicellular developmental program. eLife 8,
694 e50374. 10.7554/elife.50374

695 77. Gill, R.E., Karlok, M., and Benton, D. (1993). *Myxococcus xanthus* encodes an ATP-
696 dependent protease which is required for developmental gene transcription and
697 intercellular signaling. J Bacteriol ***175***, 4538–4544. 10.1128/jb.175.14.4538-4544.1993.

698 78. Tojo, N., Inouye, S., and Komano, T. (1993). The *lonD* gene is homologous to the *lon* gene
699 encoding an ATP-dependent protease and is essential for the development of *Myxococcus*
700 *xanthus*. J Bacteriol ***175***, 4545–4549. 10.1128/jb.175.14.4545-4549.1993

701 79. Caberoy, N.B., Welch, R.D., Jakobsen, J.S., Slater, S.C., and Garza, A.G. (2003). Global
702 mutational analysis of ntrc-like activators in *Myxococcus xanthus*: Identifying activator
703 mutants defective for motility and fruiting body development. J Bacteriol ***185***, 6083–6094.

80. Ueki, T., and Inouye, S. (2002). Transcriptional activation of a heat-shock gene,lond, of *Myxococcus xanthus* by a two component histidine-aspartate phosphorelay system. J Biol Chem ***277***, 6170–6177. 10.1128/jb.185.20.6083-6094.2003

81. Schlichting, C.D. (2021). Plasticity and Evolutionary Theory: Where We Are and Where We Should Be Going. In Phenotypic Plasticity & Evolution, pp.367–394. (CRC Press).

82. Schlichting, C.D. (2008). Hidden reaction norms, cryptic genetic variation, and evolvability. Ann Ny Acad Sci ***1133***, 187–203. 10.1196/annals.1438.010

83. Morgan, A.D., MacLean, R.C., Hillesland, K.L., and Velicer, G.J. (2010). Comparative analysis of myxococcus predation on soil bacteria. Applied and Enviro Microbiol ***76***, 6920–6927. 10.1128/aem.00414-10

84. Rendueles, O., Amherd, M., and Velicer, G.J. (2015). Positively frequency-dependent interference competition maintains diversity and pervades a natural population of cooperative microbes. Current Biology ***25***, 1673–1681. 10.1016/j.cub.2015.04.057

85. Rosenberg, E., Keller, K.H., and Dworkin, M. (1977). Cell density-dependent growth of *Myxococcus xanthus* on casein. J Bacteriol ***129***, 770–777. 10.1128/jb.129.2.770-777.1977

86. Velicer, G.J., Kroos, L., and Lenski, R.E. (1998). Loss of social behaviors by *Myxococcus xanthus* during evolution in an unstructured habitat. Proceedings of the National Academy of Sciences of the U.S.A ***95***, 12376–12380. 10.1073/pnas.95.21.12376

87. Cooper, T.F., and Lenski, R.E. (2010). Experimental evolution with *E. coli* in diverse resource environments. I. Fluctuating environments promote divergence of replicate populations. BMC Evol Biol ***10***, 11. 10.1186/1471-2148-10-11

88. Lobkovsky, A.E., and Koonin, E.V. (2012). Replaying the tape of life: Quantification of the predictability of evolution. Frontiers Genetics ***3***, 246. 10.3389/fgene.2012.00246

89. Kinnersley, M., Schwartz, K., Yang, D.-D., Sherlock, G., and Rosenzweig, F. (2021). Evolutionary dynamics and structural consequences of de novo beneficial mutations and mutant lineages arising in a constant environment. BMC Biol *19*, 20. 10.1186/s12915-021-00954-0

90. Dahl, J.L., Ulrich, C.H., and Kroft, T.L. (2011). Role of phase variation in the resistance of *Myxococcus xanthus* fruiting bodies to *Caenorhabditis elegans* predation. J Bacteriol ***193***, 5081–5089. 10.1128/jb.05383-11

734 91. Luciano, J., Agrebi, R., Gall, A.V.L., Wartel, M., Fiegna, F., Ducret, A., Brochier-Armanet,
735 C., and Mignot, T. (2011). Emergence and modular evolution of a novel motility machinery
736 in bacteria. PLoS Genet ***7***, e1002268. 10.1371/journal.pgen.1002268

737 92. Goldman, B., Bhat, S., and Shimkets, L.J. (2007). Genome evolution and the emergence
738 of fruiting body development in *Myxococcus xanthus*. PLoS One *2*, e1329.
739 10.1371/journal.pone.0001329

740 93. Schaal, K.A., Yu, Y.-T.N., Vasse, M., and Velicer, G.J. (2021). Allopatric divergence limits
741 cheating range and alters genetic requirements for a cooperative trait. bioRxiv,
742 2021.01.07.425765.

743 94. Yang, Z., and Higgs, P. I. eds. (2014). Myxobacteria: Genomics, Cellular and Molecular
744 Biology. (Caister Academic Press).

745 95. Pfennig (2021). Key Questions about Phenotypic Plasticity. In Phenotypic Plasticity &
746 Evolution - Causes, Consequences, Controversies. (CRC Press).

747 96. Bretscher, A.P., and Kaiser, D. (1978). Nutrition of *Myxococcus xanthus*, a fruiting
748 myxobacterium. J Bacteriol ***133***, 763–768. 10.1128/jb.133.2.763-768.1978

749 97. Team, R.C. (2018). R: A language and environment for statistical computing.

METHODS

Semantics. Here we specify our intended meanings of several terms in the context of this study:

- *development* (with respect to myxobacteria) - the collective set of behavioral processes leading to both fruiting body morphogenesis and sporulation.
- *divergence* - any degree of evolved genetic or phenotypic difference, whether between ancestral and derived genotypes or between distinct derived genotypes.
- *diversification* - the process by which distinct contemporary individuals or populations diverged from one another evolutionarily, genotypically and/or phenotypically.
- *latent phenotype*[29,30] - a phenotype that is potentiated by a genotype but remains unmanifested until the causal genotype is exposed to an inductive environment. See Freund et al.[30] for elaboration on use of the term 'latent-phenotype evolution' (LPE). Similar terminology has also been used recently by Kinsler et al.[31].
- *inductive environment* - an environment that induces manifestation of a phenotype that is unmanifested in other (non-inductive) environments.

MyxoEE-3. The evolved populations used in this study are a subset of populations from a broader evolution experiment named MyxoEE-3, where 'MyxoEE' stands for 'Myxobacteria Evolution Experiment' and '3' refers to the temporal rank position of the first publication from MyxoEE-3[73] relative to those from other MyxoEEs[28]. Details of MyxoEE-3 have been described previously[29,30,60,73,74] and aspects of this experiment most relevant to this study have been summarized in the Introduction. We also provide an additional summary of the manipulated differences between the selective environments of TS-B (Table S2). Replicate MyxoEE-3 populations examined here were founded by distinct sub-clones of *M. xanthus* strain GJV1[65] (here referred to as 'Anc' for ancestor), including six replicate populations each for the CTT-HA and CTT-SA treatments at cycle 40 and four each for the four treatments with prey and the CTT-HA treatment at cycle 18 (Table S1).

Induction of development. Populations of exponentially growing cultures were pelleted, the supernatant discarded, and resuspended in TPM liquid buffer[96] to a final density of ~5 x 10^9 cells/mL. Starvation plates were prepared one day prior to the experiment by pouring 10 ml of TPM agar into small Petri dishes and allowing solidification while uncovered under laminar flow. In most developmental assays, the agar concentration of TPM agar was 1.5%, but we also manipulated agar concentration to include 0.5% and 1.0% when characterizing developmental plasticity in TS-A evolved populations (Fig. 2 and Fig. S2). Consistently in all assays, 50 µl of resuspended culture (~2.5 x 10^8 cells) were spotted at the center of TPM

785 agar plates and incubated at 32 °C for 5 days before the plates were imaged and
786 morphological traits subsequently quantified.

787 **Image acquisition and trait quantification.** Starvation plates of the evolved populations
788 were imaged after 5 days of starvation for representative pictures (Figs. 1A, 3A and S3B) with
789 a Zeiss STEMI 2000 microscope and captured with a Nikon Coolpix S10 camera. Images for
790 quantitative morphological analysis were acquired using an Olympus SXZ16 microscope
791 mounting an Olympus DP80 camera system. The image-acquisition parameters were kept
792 identical in all cases (exposure time = 9.9 ms, lens = Olympus 0.5xPF, zoom = 1.25x, ISO =
793 200, illumination = BF built-in system). Images were processed and analyzed following the
794 protocol described by La Fortezza and Velicer[9]. Fruiting body (FB) morphology was
795 characterized by measuring the following traits. *FB number*: total number of fruiting bodies on
796 a single developmental plate; *Density*: grey-value intensity of pixels per FB; *Density*
797 *heterogeneity*: standard deviation of within-FB pixel-grey values (FB density); *FB area*: plate-
798 surface area occupied per FB, expressed in total pixel number. For more details about each
799 trait see La Fortezza and Velicer[9]. Median values of *Density*, *Density heterogeneity* and *Area*
800 per plate were used for further analysis. Image acquisition and trait quantification of the
801 evolved populations were always run in parallel with Anc during each biological replicate.

802 **Spore counts**. A total of ~2.5 x 10^8 cells in a 50 ul suspension (TPM liquid) were spotted on
803 each starvation plate (TPM 1.5% agar) and harvested after 5 days using a sterile spatula and
804 washed into one milliliter of ddH_2O. Samples were then heated at 50 °C for 2 hours to kill
805 vegetative cells, sonicated, diluted into CTT soft agar and incubated for 7 days before colonies
806 were counted. Also in these assays, Anc and evolved populations were assayed in parallel
807 within each biological replicate.

808 **Statistical analysis.** All experiments were performed in three temporally independent
809 replicate blocks. Each replicate consisted of the analyzed evolved populations and their
810 respective ancestral Anc sub-clones. R v4.0.0 software was used for all statistical analyses[97].
811 Spore counts were $\log_{10}$-transformed prior to statistical analysis. For those cases in which the
812 number of spores detected at the lowest dilution factor was zero (0), these counts were
813 converted to one (1) prior to the $\log_{10}$-transformation.

814 **Multivariate analysis.** Using a previously developed approach[9], FB morphology was
815 analyzed with a multivariate analysis. In brief, the obtained morphological trait values were
816 first averaged across all evolved population replicates from each selective environment and
817 across the Anc-subclones for each biological assay replicate. Then, *stat::prcomp()* function in
818 R was used to run PCA on the morphological scaled values. Plots reporting the PCA results
819 (referred as morphospace in the main text) were obtained using the *ggbiplot::ggbiplot()* (Fig.
820 1B and Fig. 3B) or *ggplot2::ggplot()* (Fig. S3C) functions. In the only case of CTT-SA PCA

results, replicate three (3) was adjusted by a small false value of 0.05 exclusively for graphical purposes in Fig 1B. This adjustment was not performed for any of the other analyses that focused on CTT-SA. After assessing homoscedasticity of the data's dispersion in the multivariate space with the *vegan::betasiper()* function followed by a post-hoc Tukey (*stats::TukeyHSD()* function), perMANOVA (*vegan::adonis()* function) was used to test whether selective environments and ancestral identity (Anc) significantly structured data's dispersion. Inter-population diversification was calculated as in La Fortezza and Velicer[9].

Cluster analysis. Cluster numbers in k-means analyses were determined by estimating the average silhouette width from the PCA results (*factoextra::fviz_nbclust()*). Once the number of optimal clusters in each case was defined (k = 2 in both TS-A and TS-B), the actual k-means analysis was performed (*stats::kmeans()*) and results plotted (*factoextra::fviz_clust()*) (Fig. S1C-E and Fig. S4C-E). In addition, hierarchical clustering analysis based on the *ward* method was also calculated from the relative Euclidean distances between all treatments' centroids mapped on the morphospace (*stats::hclust()*) (Fig. S1E, Fig. S4E).

FIGURES

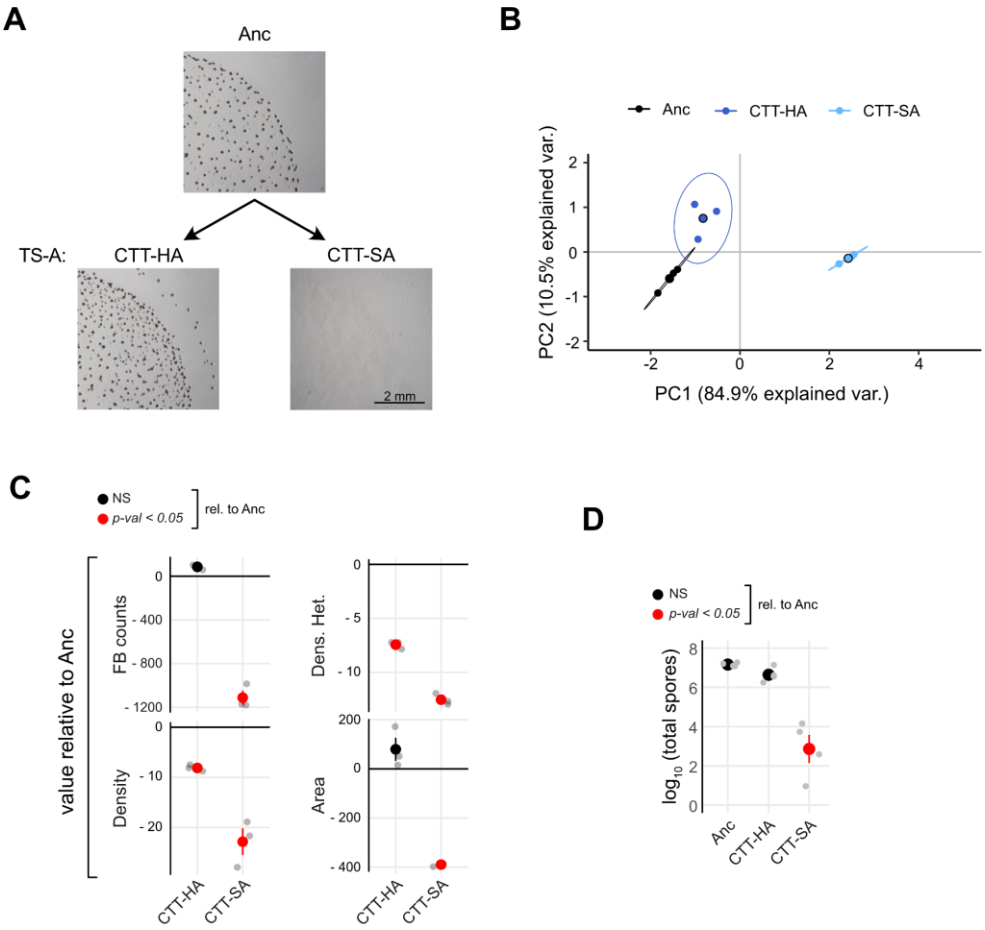

Fig. 1. Surface stiffness shapes developmental LPE.

A, Representative developmental phenotypes of the ancestor (Anc) and TS-A evolved populations from CTT-HA (P3), CTT-SA (P39) (scale bar = 2 mm). **B**, PCA based on four morphological traits showing the overall treatment-level phenotypic differentiation among the CTT-HA (dark blue) and CTT-SA (light blue) TS-A treatments (MyxoEE-3 cycle 40) and their ancestor (black). Large circles represent average morphospace localization (centroids) obtained from three independent biological replicates (small circles, n = 3). Ellipses represent 95% confidence regions, while percentage values on the x and y axis report the variation explained by the principal components PC1 and PC2, respectively (Fig. S1A). **C**, Mean values ± SEM of each analyzed morphological trait relative to the ancestral levels (Anc, black-horizontal line in the graphs) (n = 3). **D**, Mean values ± SEM of $\log_{10}$-transformed spore counts (n = 4). In both **C** and **D**, red and black circles indicate significant (p < 0.05) and non-significant (NS) differences from Anc levels, respectively. (Significance was estimated for both **C** and **D** with one-way ANOVA followed by two-tailed Tukey tests. p-values of all comparisons of evolved treatments with Anc, as well as all pairwise comparisons between evolved treatments are reported in Data Fig. 1).

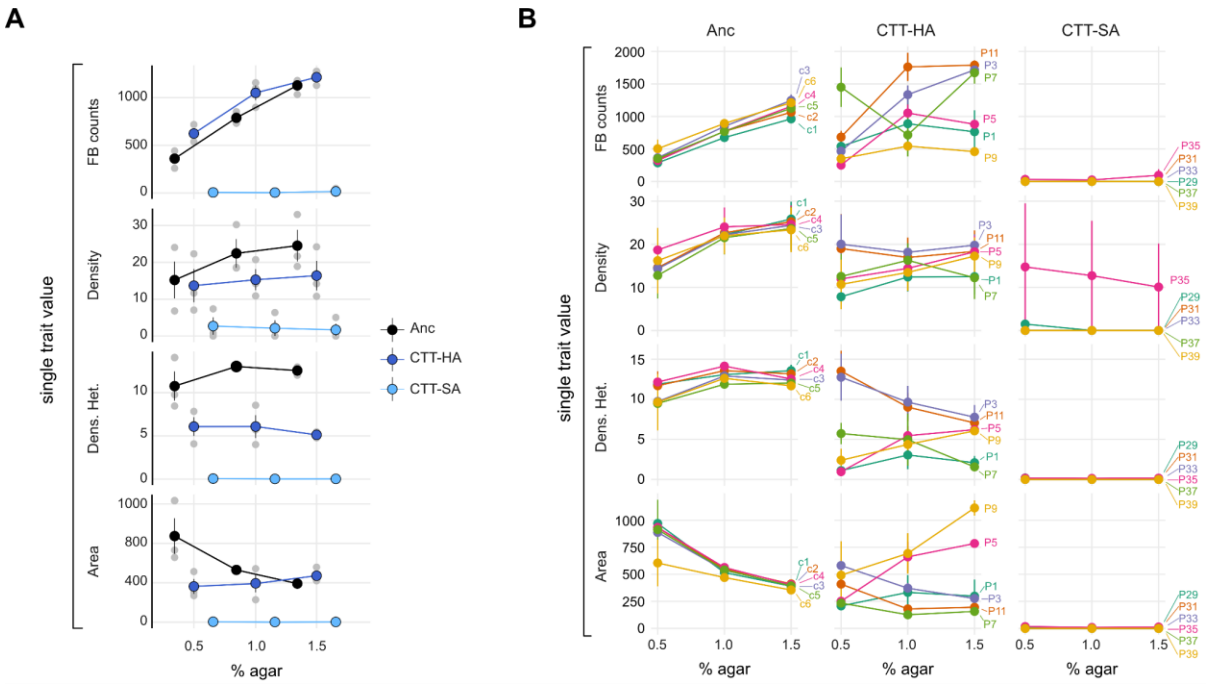

Fig. 2. Deterministic and stochastic evolution of latent reaction norms.

Reaction norms of four morphological traits across three agar concentrations (0.5%, 1.0% and 1.5%, x-axis in both panels). **A**, Reaction norms across ancestral clones (black circles and lines) and treatment-level average trait values (CTT-HA and CTT-SA evolved populations, dark and light blue circles and lines, respectively). *n* = 3 independent replicates (grey circles). **B**, Reaction norms for each individual Anc sub-clone (c1-c6) and each TS-A evolved population across the three biological replicates (*n* = 3). Labels at the end of each reaction norm indicate the identity of the ancestral clone or evolved population. In both **A** and **B**, large circles and error bars indicate the mean ± SEM, respectively. (Significance of variables' contribution and their interaction was calculated for both A and B with two-way ANOVA. Employed models and results of all comparisons are reported in **Data Fig. 2**).

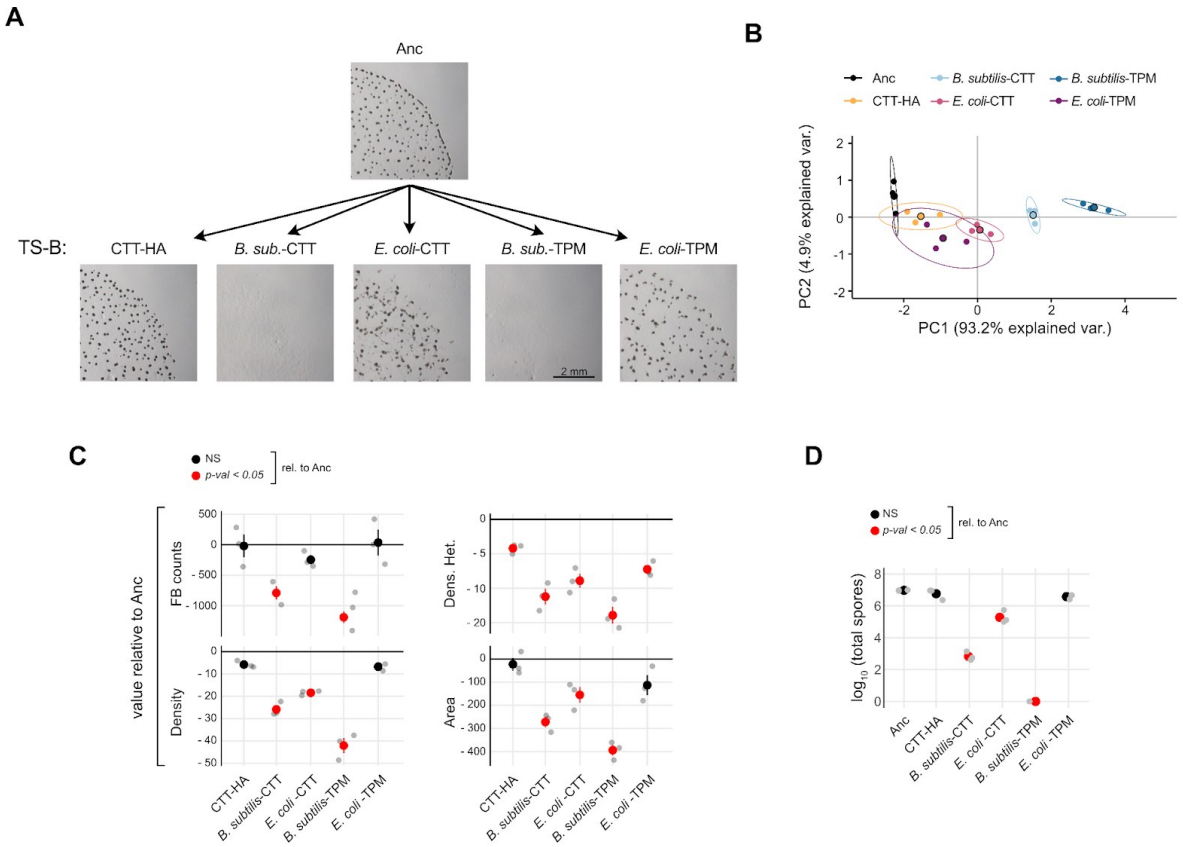

Fig. 3. Prey presence and identity shape developmental LPE.

A, Developmental phenotypes of representative TS-B evolved populations from each selective environment and Anc (CTT-HA = P1; *B. subtilis*-CTT = P99; *E. coli*-CTT = P93; *B. subtilis*-TPM = P133; *E. coli*-TPM = P127). **B**, PCA of overall morphological divergence across all five TS-B evolutionary treatments (MyxoEE-3 cycle 18) and the Anc sub-clones. Large circles represent average morphospace localization (centroids) obtained from three independent biological replicates (small circles, $n = 3$), while ellipses represent 95% confidence region. Percentage values on the x and y axis report the variation explained by the two principal components PC1 and PC2, respectively (Fig. S5B). **C**, Mean values ± SEM of individual developmental traits relative to the ancestral (Anc) levels (black-horizontal line in each graph) ($n = 3$). **D**, Mean values ± SEM of $\log_{10}$-transformed spore counts obtained after five days of starvation ($n = 3$). In both **C** and **D**, red and black circles indicate significant ($p < 0.05$) and non-significant (NS) differences from Anc levels, respectively. (Significance was calculated for both **C** and **D** with one-way ANOVA followed by two-tailed Tukey tests. *p*-values of all comparisons of evolved treatments with Anc, as well as all pairwise comparisons between evolved treatments are reported in **Data Fig. 3**).

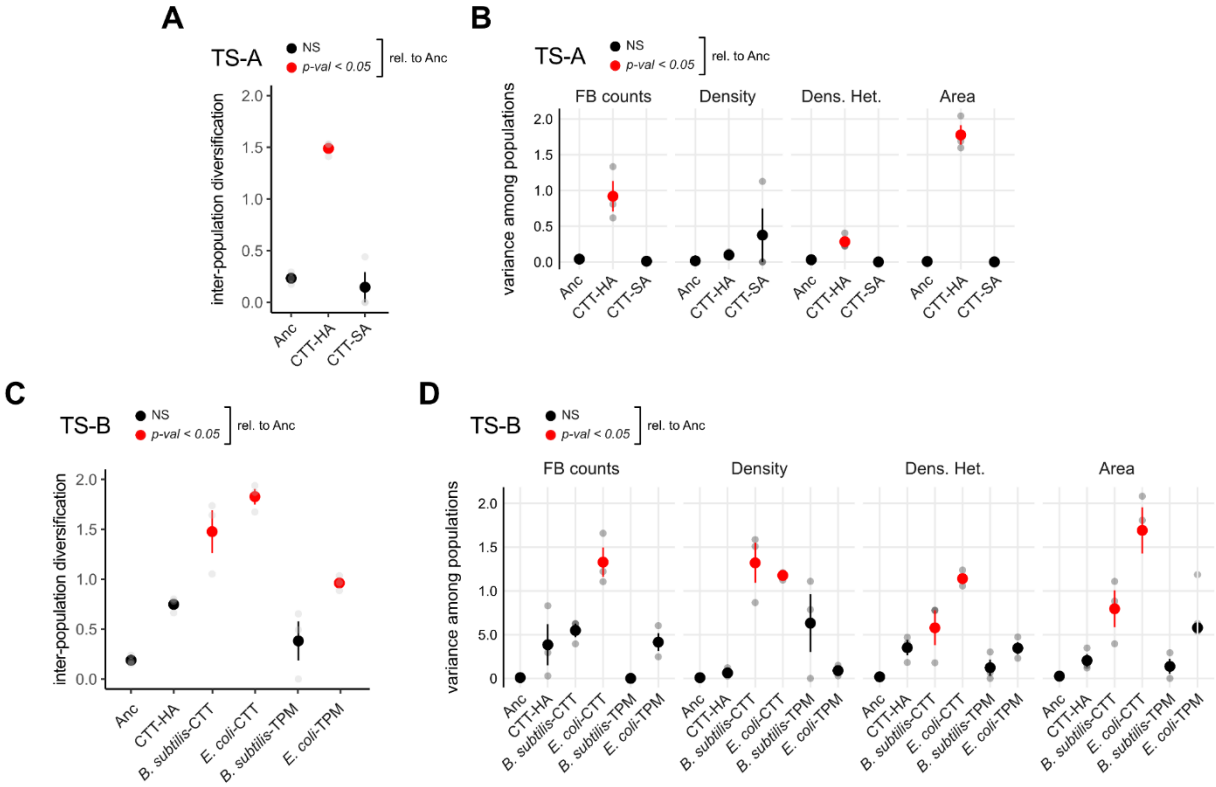

Fig. 4. Deterministic limitation of stochastic latent-phenotype diversification by MyxoEE-3 selective environments.

A and **C**, Morphological diversity among replicate TS-A (**A**) and TS-B (**C**) populations evolved in the same selective environment compared to diversity among Anc sub-clones. **B** and **D**, Variance of single morphological traits distributions calculated across TS-A (**B**) and TS-B (**D**) evolved populations and across the Anc sub-clones. In all cases, large circles represent mean values ± SEM of three independent biological replicates (grey circles, n = 3), while red and black circles indicate significant (p < 0.05) and non-significant (NS) differences from Anc levels, respectively. (Significance was calculated in all cases with one-way ANOVA followed by two-tailed Tukey tests. p-values of all comparisons of evolved treatments with Anc, as well as all pairwise comparisons between evolved treatments are reported in Data Fig. 4).

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