

Overshooting dynamics in a model adaptive radiation

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The history of life is punctuated by repeated periods of unusually rapid evolutionary diversification called adaptive radiation. The dynamics of diversity during a radiation reflect an overshooting pattern with an initial phase of exponential-like increase followed by a slower decline. Much attention has been paid to the factors that drive the increase phase, but far less is known about the causes of the decline phase. Decreases in diversity are rarely associated with climatic changes or catastrophic events, suggesting that they may be an intrinsic consequence of diversification. We experimentally identify the factors responsible for losses in diversity during the later stages of the model adaptive radiation of the bacterium *Pseudomonas fluorescens*. Proximally, diversity declines because of the loss of biofilm-forming niche specialist morphotypes. We show that this loss occurs despite the presence of strong divergent selection late in the radiation and is associated with continued adaptation of resident niche specialists to both the biotic and abiotic environments. These results suggest that losses of diversity in the latter stages of an adaptive radiation may be a general consequence of diversification through competition and lends support to the idea that the conditions favouring the emergence of diversity are different from those that ensure its long-term maintenance.

Keywords: adaptive radiation; divergent selection; niche specialization; diversity; adaptation; *Pseudomonas fluorescens*

1. INTRODUCTION

Adaptive radiation is the rapid diversification of a single lineage into a range of ecologically and phenotypically distinct niche specialists [1–4]. The rate at which a lineage diversifies depends on the difference between speciation and extinction rates. In the initial stages of adaptive radiation, speciation rates far exceed extinction rates owing to the combined effects of strong diversifying selection, generated typically through either resource competition or predation, and abundant ecological opportunity (vacant niche space or unused resources). As the radiation proceeds, ecological opportunities become filled and diversification slows owing to declining speciation rates [5,6]. Models of this process produce an S-shaped relationship between species richness and time that closely resembles the logistic population growth curve in population biology [7], with the equilibrium level of species diversity set by the extent of ecological opportunity. Contrary to this expectation, however, evidence from both the fossil record [8,9] and a number of extant radiations including tetragantheid spiders [10], Darwin's finches [11] and African cichlid fish [12] indicate that species richness often declines in the later stages of many radiations, a phenomenon sometimes termed 'overshooting' [13]. Overshooting must be the result of either speciation rates declining below extinction

rates, or extinction rates increasing, but the underlying causes of overshooting have not been systematically explored.

At least three hypotheses could explain why radiations tend to lose diversity as they age. First, newly formed species with small populations are prone to extinction [14]. Second, environmental degradation caused by pollution and the exhaustion of nutrients may either reduce the extent of ecological opportunity itself, leading to the loss of susceptible niche specialists, or decrease population density, which can weaken resource competition and so the strength of divergent selection [15]. Third, the strong divergent selection responsible for diversification early in the radiation may persist into its later stages leading either to the loss of ecologically intermediate types with lower fitness than more extreme types [16] or to the competitive replacement of variants within a niche class (ecomorphs; [10]). Note that under both scenarios strong divergent selection acts to ensure coexistence of distinct niche specialists. This third hypothesis has been proposed to explain the dynamics of diversity in Trilobites [17] and African cichlid fish [12]; species diversity was lost in the later stages of the radiation but phenotypic disparity (a measure of the total phenotypic variance among ecologically relevant traits, and so a proxy for ecological divergence) between surviving lineages continued to increase. This result suggests that ecologically intermediate types were out-competed by more ecologically divergent forms.

To understand the mechanisms responsible for the loss of diversity following adaptive radiation, we have studied

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the dynamics of diversity in static microcosms of the soil bacterium *Pseudomonas fluorescens* (strain SBW25), which has become widely used as an experimental model of adaptive radiation. The ecological and genetic processes underlying morphotype diversification in this system have been well studied [18–21]. Briefly, population growth of the broth-colonizing ancestor in unshaken microcosms generates strong resource competition for limiting resources such as oxygen which, in the presence of ecological opportunity afforded by spatial structure, leads to strong diversifying selection. Recurrent mutation introduces a constant supply of ecologically and genetically distinct variants resulting in the rapid emergence of a range of niche specialist genotypes that can be readily identified by eye when grown on agar plates. The three main classes of niche specialists are the ancestral broth-colonizing *smooth* (SM), the biofilm-forming *wrinkly-spreader* (WS; together these two classes typically comprise well over 95 per cent of all morphotypes in our experiments) and *fuzzy-spreaders* (FZ). We refer to these types as ecomorphs in what follows. Coexistence among ecomorphs is supported by negative frequency-dependent selection [15,18]. Unique mutations can often give rise to a range of phenotypically similar genotypes within each ecomorph [20,21], which can be further differentiated by the range of sugars they metabolize [22].

Our experiments track diversity as the number of distinct morphotypes, or richness, over time. This measure thus includes both the striking niche specialization of SM, WS and FZ morphologies that is associated with spatial structure in these microcosms as well as all morphologically distinguishable types that arise within each of these three ecomorphs. Morphological variation within an ecomorph has been tied previously to functional differences in resource use [22] and genetic variance in fitness [20,23], suggesting that these types may be further ecologically differentiated within their respective niches as well. We obtained a second measure of diversity based on resource differentiation using metabolic profiles (following [22]). In practice, distance-based metrics based on metabolic profiles consistently group SM and WS morphotypes as distinct clusters (see electronic supplementary material) and provide a finer-scale picture of ecological differentiation among variants within each ecomorph in our experiment. The number of distinct metabolic profiles thus constitutes an alternative measure of diversity that can also be used to express the quantitative extent of metabolic differentiation that evolves over the course of a radiation. Finally, because reproduction in this system is asexual, morphotype diversity here is formally equivalent to species diversity when reproduction is sexual [24].

2. MATERIAL AND METHODS

(a) *Experimental overview*

Our experiments were performed in a top-down hierarchical structure: first we documented the pattern of overshooting (§2*b,c*). Next we tested the hypotheses that either abiotic or biotic environmental changes cause the decline in overall community diversity (§2*d*). Finally, we hone in on how interactions between competing individuals drive the patterns of decline we observed (§2*e,f*). Together, these studies combine to provide a comprehensive analysis of how a

macroevolutionary pattern develops from micro-scale interactions between individual genotypes.

(b) *Dynamics of diversity*

Initially isogenic populations of *P. fluorescens* SBW25 with the neutral *lacZ* genetic marker [21] were allowed to diversify for 10 days in 6 ml of King's B (KB) medium, following previous work [18], and destructively sampled every 8 h for the first 4 days and then daily for the remaining 6 days. Five replicate microcosms were sampled at each time point. Samples of entire populations were frozen in 20 per cent glycerol. Diversity was estimated as morphotype richness (number of distinct colony morphs on an agar plate) and metabolic profile richness (number of distinct metabolic profiles assessed using BIOLOG GN2 microwell plates; see §2*c*). We recorded morphotype richness as the number of unique morphotypes observed by plating diluted cultures on KB agar and counting 100 colonies. Metabolic diversity would ideally be assayed in a similar way, however the large numbers of BIOLOG plates required makes this impractical. Instead, we restricted attention to the BIOLOG profile of SBW25 and all phenotypically distinct clones from communities sampled at 48, 96 and 200 h. In all, 24 genotypes were isolated. Metabolic richness was measured by counting the number of distinct metabolic profiles in a community at a given time. We also measured metabolic disparity by calculating the average Euclidean distance in metabolic profiles between the ancestor and all co-occurring genotypes at a given time point using the `dist()` function in R [25].

(c) *BIOLOG assays*

Following previous work [22], we grew a single colony of each isolated genotype overnight in 5 ml of M9KB at 28°C shaken at 200 r.p.m. and then starved cells by transferring 20 µl of the dense culture to 20 ml of M9 minimal salts and incubating at 28°C on an orbital shaker for approximately 2 h. To each well on the BIOLOG plate, we transferred 150 µl of the starved cells. Assays of the ancestral genotype were performed in triplicate to obtain an estimate of error variance; evolved morphotypes were assayed once. We used the error estimate (Euclidean distance of 0.85) as a minimum threshold value to distinguish whether the evolved genotypes were in fact distinct; clones more similar than 0.85 were lumped together. This approach allows us to obtain profiles for more genotypes and is justified by previous work showing that assays from replicate cultures read on the same day are highly repeatable [22]. Absorbance was read just after inoculation and again after 48 h as optical density at 630 nm (OD_{630}) with a Universal Microplate Reader (ELx800, Bio-Tek Instruments Inc.). The final OD_{630} used in the analysis was corrected for the initial OD_{630} taken just after inoculation and an estimate of the error variance associated with replicate readings of the microplate reader: $\Delta OD_{630} = OD_{630}(48\text{ h}) - OD_{630}(0\text{ h}) - 2 \times \text{s.d. of blank wells}$. Corrected absorbance readings served as our measure of performance on each substrate.

(d) *Whole-community transplant experiment*

To test the hypothesis that environmental degradation drives the loss of diversity in the later stages of the radiation, we reciprocally transplanted entire communities of bacteria isolated from early (2-day) and late (8-day) phases of the radiation into both fresh and 8-day used medium (spent) and recorded changes in diversity after 2 days. Communities were separated from the medium by centrifugation and the

medium was cleared of all cells by filtration and then individually conditioned for 4 h in M9 minimal salts [26]. Each community was then inoculated into microcosms containing 6 ml of either 2- or 8-day spent medium. A fully factorial experiment was conducted with three replicate microcosms for each community–growth medium combination. Morphotype richness was measured at initiation and 2 days later.

(e) *Estimating frequency-dependent fitness functions*

We estimated the frequency-dependent fitness functions between 16 separate pairs of WS and SM, two pairs being isolated from each of four separate microcosms sampled at 48 h and another four microcosms sampled at 200 h, to test the hypothesis that divergent selection remained strong throughout the experiment. We intentionally chose phenotypically distinct WS morphotypes from the same microcosm in order to ensure that each WS–SM pair represented a unique combination. For each SM–WS pair, a total of 12 microcosms were inoculated with different initial ratios of SM versus WS. Additionally, 48 h clones were competed in fresh medium and 200 h clones were competed in spent medium to simulate the environmental conditions the clones would experience during the radiation. Each microcosm was initiated at a total density of approximately 1×10^8 cfu ml⁻¹. Before inoculation, each strain was grown overnight in 6 ml of KB at 28°C shaken at 150 r.p.m. and then acclimated to the experimental medium for 4 h. Microcosms were sampled at initiation and 48 h later as described previously [18]. The selection coefficient was calculated for each microcosm as $r = (\ln[SM_{\text{final}}/SM_{\text{initial}}] - \ln[WS_{\text{final}}/WS_{\text{initial}}]) / \text{time}$ (as in [27]). A general linear model (GLM) was performed with the program JMP to test the effects of initial frequency on SM fitness early and late in the radiation: $r_i = y_0 + \text{freq.} + \text{time} + \text{freq.} \times \text{time} + \text{microcosm}[\text{time}] + \text{pairs}[\text{microcosm}, \text{time}] + \varepsilon_i$. Here, r_i is the relative fitness of a SM in competition with a WS, which is a function of its frequency (freq.), the community from which the pairs were isolated (time; early or late), and an interaction between the two (freq. \times time). Because the sampling procedure involved removing SM–WS pairs from independent microcosms at each time point, we also asked whether we could detect variation among microcosms within a time point (microcosm nested within time: microcosm [time]), and among pairs within a microcosm (pairs nested within microcosm and time: pairs[microcosm, time]). Our interest here is specifically in the significance of the interaction term: if the strength of divergent selection among ecomorphs remains the same throughout the radiation, then the frequency-dependent fitness functions will be indistinguishable for ‘early’ and ‘late’ microcosms as revealed by a non-significant interaction term in the GLM.

(f) *Niche-specific adaptation*

We also tested whether evolved niche specialists from late in the radiation showed evidence of continued adaptation using four-way competition experiments involving evolved and ancestral strains of both SM and WS in the same microcosm. Competition experiments were between a single-derived WS and derived SM from a *lacZ* neutrally marked SBW25 [21], with the unmarked ancestor SBW25 and an unmarked WS (termed the *large-spreading wrinkly-spreader* or LSWS for short, previously isolated by Goymer *et al.* [19]. LSWS differs from SBW25 by a single point mutation in *wspF* [20] and has a characteristic phenotype that allows us to

distinguish it by eye from the evolved WS morphotypes in our experiment. Second-site mutations that improve fitness without compromising the ability to produce a biofilm are expected to arise through continued evolution in static microcosms, thus allowing us to use the LSWS as a standard competitor strain to assess adaptation among WS. All genotypes were initiated at approximately equal frequencies and fitness calculated as the change in ratio of the evolved niche specialist and its ancestral counterpart. This design ensures that both the broth and air–broth interface are occupied during competition, which prevents diversification into the alternate niche and improves reproducibility of fitness assays (see [20]). Two sets of competition experiments were assembled; derived clones isolated at 48 h (early) and clones isolated from 200 h (late). Four derived clones of each type were isolated from unique microcosms. Competition experiments were assembled, run and fitness was computed identically to the frequency-dependent fitness experiments except the frequencies of each clone were kept uniform between the replicates. Additionally, only three replicates for each competition were performed. Competition experiments were sampled by plating on X-gal indicator plates [21] to distinguish between the derived and ancestral clones. To determine whether there was an effect of time on the evolved genotypes fitness, we performed a GLM with the program JMP: $r_i = y_0 + \text{time} + \text{microcosm}[\text{time}] + \varepsilon_i$. The fitness of an evolved niche specialist relative to its less-derived niche counterpart (r_i) is a function of when the evolved specialist was isolated (time), while controlling for any effect each particular microcosm may have on fitness (microcosm nested within time: microcosm[time]).

3. RESULTS

(a) *Dynamics of diversity*

The overall dynamics of diversification in our experiment closely resemble those seen in many natural radiations, with a rapid exponential-like increase in morphotype richness initially followed by a slower loss (figure 1). Diversity, estimated by both colony morphology richness and the number of distinct metabolic profiles, is highest at day 4, when all three niche-specialist classes are present. Thereafter, diversity declines because of the loss of morphotypes within the WS class (electronic supplementary material, figure S1b) and genotypes with metabolic profiles less divergent from the ancestor. Consistent with the hypothesis that overshooting stems from the loss of types with ecologically intermediate resource requirements, disparity from the ancestral metabolic profile continues to increase throughout the radiation even as morphotype richness wanes (figure 1b; electronic supplementary material, figure S1c). Notably, all three niche-specialist morphotypes persist at roughly the same frequency through the entire experiment (electronic supplementary material, figure S1c), suggesting that negative frequency-dependent selection remains strong late in the radiation and the loss of diversity in the later stages stems from selection among genetically distinct variants within the WS class. The loss of morphotype and metabolic richness is also accompanied by a modest decrease in total population density from 5×10^8 colony forming units (cfu) per millilitre after 1 day to approximately 1×10^8 cfu ml⁻¹ by day 10 owing to the depletion of resources and pollution by secondary metabolites.

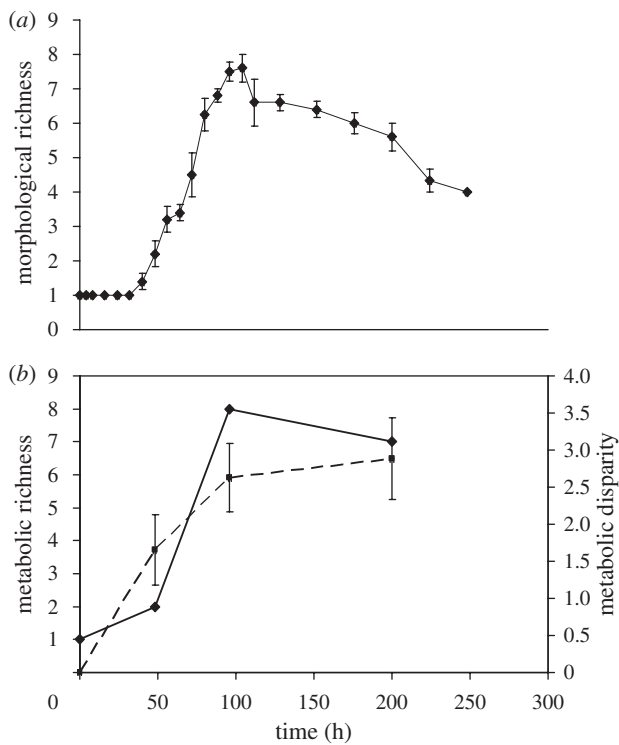


Figure 1. Dynamics of radiation. (a) Mean morphological richness; (b) total metabolic richness (diamonds and solid line; see text) and metabolic disparity (squares and dashed line; see text) over the course of the radiation. Error bars represent ± 1 s.e.m.

(b) The causes of overshooting

Proximately, the loss of morphotype richness in the later stages of this radiation is because of the extinction of morphotypes within the WS class (electronic supplementary material, figure S1). This process is unlikely to be caused by stochastic effects such as drift because population sizes remained large throughout the experiment (on the order of 10^8 cells) and similar morphotypes go extinct in replicate populations.

The modestly smaller population sizes at the end of the experiment suggest that environmental degradation could be responsible for the loss of WS morphotype diversity in our experiment. To test this idea directly, we conducted a whole-community reciprocal transplant and followed the changes in morphotype diversity that resulted. If environmental degradation is the cause of the decline phase, then both early and late communities will lose diversity in the spent medium. The response of diversity depended on both the age of the community and the environment in which it was cultured (figure 2), as revealed by a significant community–media interaction in an analysis of variance ($F_{2,16} = 8.64$, $p = 0.01$). Specifically, early communities, which were somewhat less diverse than late communities initially, showed a tendency to continue diversifying in both fresh and spent medium, suggesting that resource competition is sufficiently strong and ecological opportunities sufficiently abundant to generate diversifying selection even in degraded environments. Late communities, by contrast, showed no change in diversity in fresh medium and a notable loss of diversity in spent medium. Proximately this loss of diversity stems, as before, from a reduced richness of WS morphotypes. Thus, environmental degradation alone cannot

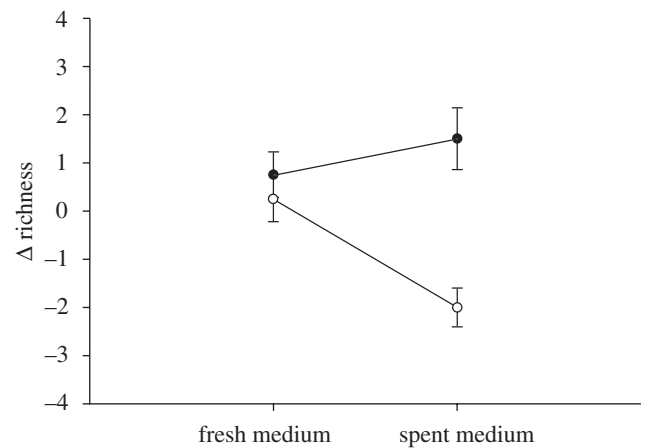


Figure 2. Dynamics of morphotype diversity in a full factorial transplant experiment. Filled circles represent early (2-day) communities, while open circles are late (8-day) communities. Each point is the average of three replicates. Error bars represent ± 1 s.e.m.

explain the decline phase. Whether diversity is lost or maintained depends on the genetic composition of the community itself.

These results point towards the third hypothesis, persistent divergent selection, as the probable cause of the decline phase in the *P. fluorescens* radiation. This hypothesis makes two compelling predictions that we can test. The first is that divergent selection, which is known to drive the emergence of diversity in the initial phases of the radiation, should remain strong in the later stages as well. The second prediction is that the persistence of strong divergent selection throughout the radiation leads to adaptation to a given niche, such that variants within an ecomorph isolated from late in the radiation will out-compete those of the same ecomorph isolated from earlier in the radiation.

We tested the first prediction, that divergent selection remains strong throughout the entire radiation, by measuring frequency-dependent fitness functions between independently evolved pairs of SM and WS at different stages of the radiation. Two SM–WS pairs were isolated from each of four independent ‘early’ and ‘late’ microcosms and competitive fitness measured in either fresh or spent medium, respectively, in order to account for the effects of environmental change. Our results (figure 3) are compelling: the frequency-dependent fitness functions for early and late pairs were identical (electronic supplementary material, table S1) and there is no significant interaction between time (early versus late) and frequency in an analysis of covariance (ANCOVA; $F_{1,172} = 0.499$, $p = 0.481$). These results suggest that divergent selection, which is known from previous work to be strong early in the radiation [15,18] remains strong in its later stages. Note that variation in the strength of frequency-dependent selection among independent microcosms was not formally significant (ANCOVA with microcosm nested within time: $F_{7,8} = 3.10$, $p = 0.07$), although we did detect such variation among genotype pairs within a microcosm (ANCOVA with genotype pairs nested within microcosm and time: $F_{8,173} = 8.21$, $p < 0.0001$), reflecting the existence of genetic variation in fitness within niche classes.

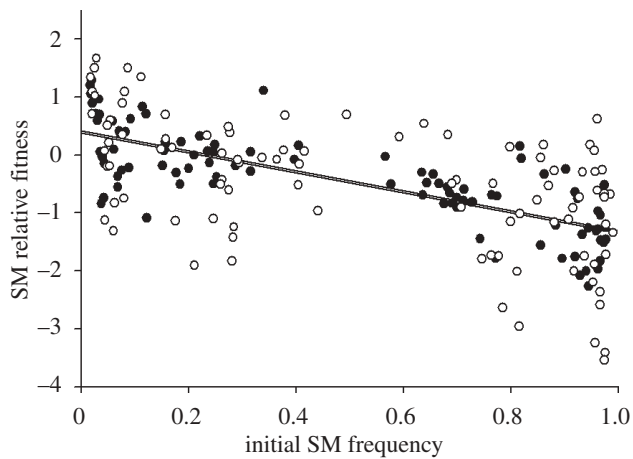


Figure 3. Frequency-dependent fitness functions between smooth and wringly spreader pairs isolated from early and late in the radiation. Each point represents the outcome of a single competition at different starting frequencies between smooth and wringly pairs isolated from early (filled circles) or late (open circles). The two estimates of frequency-dependent selection are statistically indistinguishable.

To test the second prediction of adaptation by ecomorph variants to conditions within a niche, we estimated the fitness of SM and WS isolates evolved from both early and late in the radiation in four-way competition experiments against the ancestral SM (SBW25) and the WS (LSWS). Our results indicate that WS isolated late in the radiation had higher fitness, on average, than those isolated earlier (figure 4*a*; time: $F_{1,16} = 22.35$, $p = 0.0002$) with no detectable variation among microcosms ($F_{6,16} = 1.160$, $p = 0.3743$). The same pattern was observed for SM (figure 4*b*; time: $F_{1,16} = 7.956$, $p = 0.0123$; microcosm: $F_{6,16} = 1.680$, $p = 0.1898$). Thus, divergent selection has led to continued adaptive differentiation of genotypes within each ecomorph over the course of the radiation.

4. DISCUSSION

We found that the dynamics of diversity in static microcosms of *P. fluorescens* follows a pattern similar to the overshooting dynamics observed in both fossil and extant radiations. Diversity, whether measured in terms of richness of colony morphology or metabolic profiles, increases in an exponential-like fashion initially and then declines at a slower rate in the later stages of the radiation. Proximately, this loss of diversity is associated with the selective extinction of genotypes from within a given ecomorph rather than the loss of entire ecomorphs altogether. We have explored the mechanisms responsible for the loss of diversity and have shown that they involve selective replacement among genotypes within the same ecomorph. Moreover, this selective replacement is associated with the maintenance of metabolic disparity despite the loss of diversity when measured both as morphological and metabolic profile richness.

Taken together, these results provide compelling support for the idea that decreases in species diversity in the latter stages of adaptive radiations may be driven by the same mechanism that allows a radiation to proceed in the first place, namely, strong divergent selection. In the earliest stages of the radiation when vacant niche

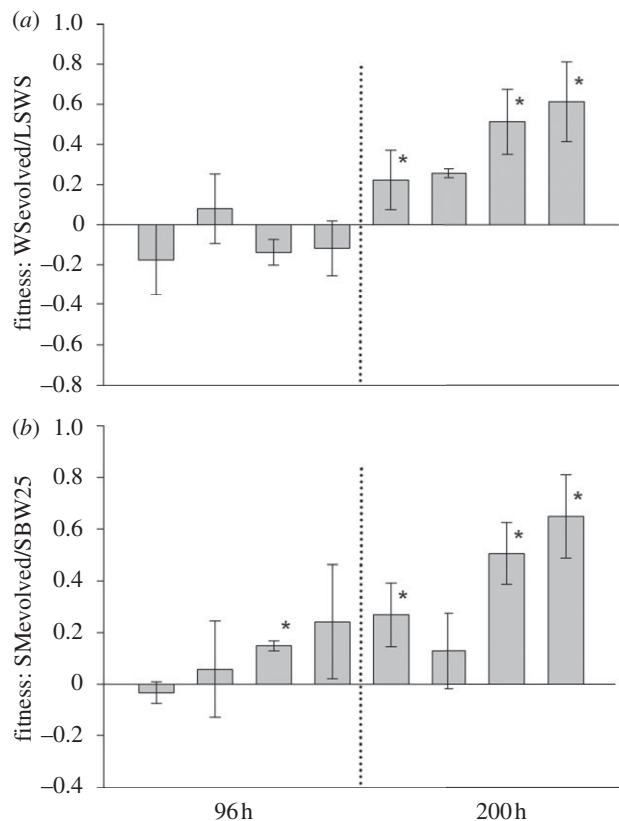


Figure 4. Outcome of four-way competition experiments between clones isolated from 96 or 200 h against ancestral clones. Each bar represents the average relative fitness for a derived genotype relative to an ancestral genotype of the same ecomorph ($n = 3$, error bars represent 1 s.e.m.). Asterisks indicate which estimates are significantly greater than 0. (a) Wringly-spreader and (b) smooth.

space is abundant, divergent selection in combination with resource competition results in diversification leading to the emergence of a range of novel niche specialists. Once ecological opportunities have been filled, however, divergent selection may still be strong but further ecological diversification is prevented owing to the presence of other niche specialists [28]. If sufficiently strong, divergent selection will support extant diversity and may even promote further adaptive differentiation among ecologically similar variants leading to the loss of inferior within-niche variants. The consequences of diversifying selection will thus depend on the extent to which ecological opportunities have been filled.

Interestingly, recent theory suggests that a similar sequence of stages should characterize many adaptive radiations [29]. Divergent selection in the presence of ecological opportunity is thought to promote a rapid burst of diversity into niche specialists that occupy major macro-habitats. Further diversification results from adaptation to micro-niches. However, as ecological opportunities fill, the consequence of divergent selection shifts from promoting ecological diversification to the selection of traits that promote the long-term survival and reproduction of lineages within niches. Adaptations accumulated in this later phase of the radiation are thought to allow some lineages to outcompete others, causing a slow decline in diversity as less-derived lineages are gradually excluded. Notably, this sequence of events is

supported by observations of species diversity on Hawaiian islands, where islands of intermediate age have high species diversity that share similar ecomorphs and older islands have only one species of each ecomorph [30]. Our results also closely resemble these patterns: we observed an initial period of rapid diversification into spatial niches (macro-habitats) followed by further differentiation into types with distinct resource use (micro-habitats) and then the eventual loss of less-derived types that overlap in their spatial niches.

Despite this apparent similarity, some may question the generality of our work because it was performed in long-term batch cultures that lack resource renewal and waste removal, whereas most 'real' radiations occur in environments where nutrients are regularly replenished and recycled. This criticism seems unfounded for two reasons. First, previous work with the same strain has shown that prolonged serial transfer generates diversity dynamics that closely resemble those reported here, with an initially rapid increase in diversity followed by a slower decline [31]. Interestingly, diversity was lost more slowly when compared with our experiment, suggesting that the resource renewal afforded by serial transfer may retard, but not prevent, extinction. Second, even depleted environments in our experiment contain sufficient nutrients, generated either as by-products of metabolism or cell lysis, to support on average 1.72 ± 0.16 s.e.m. doublings over 48 h in spent medium. Thus substantial population turnover, and so adaptation, may occur even in the absence of serial transfer [32]. Our observation of adaptive differentiation in SM and WS isolated from late in the radiation lends support to this idea and suggests that stationary phase is likely more physiologically and evolutionarily dynamic than has been previously recognized. Nevertheless, we recognize that the match between the conditions of our experimental system and those in nature may not be perfect, and our results should be interpreted with appropriate caution.

To the extent that our results are general, however, they lend support to the idea that the conditions favouring the emergence of diversity may often be substantially different from those ensuring its long-term maintenance. The implication of this view is that, in the absence of alternative diversity-supporting mechanisms such as mutualism, the long-term fate of diversity arising through adaptive radiation may ultimately be extinction for many lineages. Moreover, the overshooting dynamics of diversity during adaptive radiation underscore the highly dynamic nature of the adaptive landscape, one whose topography is difficult to predict *a priori* because it depends on the combined effects of physical structure and community composition.

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