Selection for predator resistance varies with resource supply in a model adaptive radiation

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ABSTRACT

Background: The bacterium Pseudomonas fluorescens diversifies in static laboratory microcosms, giving rise to distinct broth-colonizing ‘smooth’ and biofilm-forming ‘wrinkly spreader’ morphotypes. Growth in the biofilm confers predator resistance, but is energetically costly and therefore constrained by both the nutrient concentration of the growth medium and competition for those nutrients with the smooth population.

Question: Does the role of predation during adaptive diversification depend upon resource availability?

Organisms: Laboratory populations of Pseudomonas fluorescens and its protozoan predator Tetrahymena thermophila.

Methods: Experimental evolution of bacteria across a gradient of resource supply in the presence and absence of predators. We recorded the effects of predation and resource supply on the frequencies of resistant and susceptible morphotypes at the end of adaptive diversification. Given that there are distinct subclasses within the principal morphological categories, we also recorded changes in total phenotypic diversity.

Results: In resource-poor conditions, the broth-colonizing morphotype is preponderant, but as resource supply increases, the frequency of the biofilm-forming morphotype rises. Predation increased the frequency of wrinkly spreaders, with the largest effect at intermediate resource concentrations.

Conclusions: Predation extends the range of concentrations that support high phenotypic diversity. Co-existence between different prey phenotypes is more likely at intermediate concentrations than at extremes where one type or the other is in the majority.

Keywords: adaptive radiation, competition, experimental evolution, predation, Pseudomonas fluorescens, Tetrahymena thermophila.

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INTRODUCTION

Selection imposed by natural enemies, including predators and parasites, and resource competition are important determinants of community structure and potential drivers of diversification (Levene, 1953; Rosenzweig, 1995; Doebeli and Dieckmann, 2000; Schluter, 2000; Abrams, 2003). However, the way that these factors interact in different environments remains unclear (Abrams, 2000; Chase et al., 2002). Here we explore the roles of resource competition and predation during adaptive radiation in environments with different levels of resource availability.

Adaptive radiation, the rapid diversification of a lineage into a range of niche specialists, may be driven by diversifying selection generated through resource competition in the presence of ecological opportunity (Schluter, 2000). Predators may affect this process in a number of ways (Chase et al., 2002). For example, predation can create additional ecological opportunities in the form of predator-resistant phenotypes (Vamosi, 2005; Nosil and Crespi, 2006), which may lead to greater diversity than observed under resource competition alone. Alternatively, diversity may be reduced under predation if there is strong selection for a single resistance strategy, or if reduced prey population density reduces the strength of diversifying selection resulting from resource competition (Buckling and Rainey, 2002; Meyer and Kassen, 2007).

In the absence of predators, diversity is known to vary with environmental resource availability (Tilman, 1982; Rosenzweig, 1995), and is often seen to peak at intermediate levels (Abramsky and Rosenzweig, 1984; Abrams, 1995; Rosenzweig, 1995). In support of this general pattern, experimental studies with microbes have shown that evolutionary diversification can be constrained in low-resource environments where, in spite of resource competition, ecological opportunity is limited and the only phenotypes that persist are those that can grow at low resource concentrations (Kassen et al., 2000; Hall and Colegrave, 2007). As resource supply increases, resource competition may generate diversifying selection, leading to the evolution of niche specialists that exploit different resources or spatially defined habitats. At very high resource supply, a single phenotype often dominates, exploiting only the most productive resource or niche.

In the presence of predators, any change in diversity resulting from the evolution of predator-resistant phenotypes may be constrained by resource supply in a similar way. Specifically, if predator resistance incurs reduced growth rate or competitive ability, the selective benefits conferred by resistance may be lowest when resources are in short supply, with selection instead favouring phenotypes that are more successful at competing for the available resources (Leibold, 1996). In contrast, when resources are more abundant, resource exploitation is less important for fitness and selection is expected to favour predator resistance (Holt et al., 1994; Leibold, 1996). Co-existence between resistant and susceptible populations may therefore be most likely at intermediate levels of resource supply. Experimental evidence from Escherichia coli in the presence of bacteriophage suggests that this is the case in an ecological setting (Bohannan and Lenski, 2000).

The theoretical and experimental results outlined in the preceding paragraph lead us to predict that selection for predator-resistant phenotypes during an adaptive radiation will vary with resource availability, with energetically costly resistance strategies being less advantageous at low resource supply. In turn, we expect that any increase in diversity resulting from predation will be relatively small in these environments. We tested this hypothesis using a predator–prey system comprising the bacterium Pseudomonas fluorescens and a filter-feeding protozoan Tetrahymena thermophila. Protozoan predation of bacteria is extremely common and bacteria employ a wide range of resistance strategies...
Pseudomonas fluorescens has been used previously to show that both predation and resource competition can promote adaptive diversification (Rainey and Travisano, 1998; Meyer and Kassen, 2007), albeit in different ways. Resource competition promotes rapid diversification to spatially defined niches in static microcosms, with the principal morphological classes occupying either the liquid broth phase (ancestral smooth: SM) or the air–liquid interface (wrinkly spreader: WS) due to the formation of a self-supporting biofilm. This diversity is stably maintained by frequency-dependent selection (Rainey and Travisano, 1998). Predation by T. thermophila also promotes diversification of SM to WS due to the ability of the WS morph to escape predation in the biofilm (Meyer and Kassen, 2007).

Biofilm formation is energetically costly, requiring the over-production of a cellulosic polymer (Spiers et al., 2003; MacLean et al., 2004), and is therefore severely compromised when resources are limited. Consequently, the success of WS varies considerably with resource availability in the absence of predators (Kassen et al., 2000), being very low in resource-poor conditions and higher when resources are abundant and the energetic cost of biofilm formation is outweighed by the benefits of growth at the oxygen-rich air–liquid interface. We anticipated that the effect of predation would be to increase the relative fitness of WS at all levels of resource supply, reflected by greater WS frequency at the end of diversification. Given the energetic cost of growth at this niche, and the aforementioned evidence that selection for costly resistance strategies is relatively weak in resource-poor conditions (Leibold, 1996; Bohannan and Lenski, 2000), we predicted that the increase in WS frequency resulting from predation would be smallest in low-resource environments. Furthermore, because there are several distinct subclasses within both SM and WS, we expected changes in the frequencies of the principal morphs to have significant effects for total phenotypic diversity.

We first show how predation affects the frequencies of different morphotypes across a gradient of nutrient supply, and how this determines the level of phenotypic diversity at the end of adaptive diversification. We did this by allowing P. fluorescens to diversify in static liquid microcosms at different nutrient concentrations both with and without predation by T. thermophila. Second, we tested whether changes in the frequencies of the principal morphotypes are due to the effect of predation on the productivity of the biofilm niche compared with the broth phase, or on competition between the SM and WS populations. To do this we carried out growth assays in pure and mixed culture (different morphotypes grown in different vials or the same vial respectively) at three levels of resource supply both with and without predation. Comparing frequencies in pure and mixed culture reveals the effect of competition, so that reduced WS frequency in mixed culture suggests a negative effect of competition between populations on the relative fitness of WS.

**MATERIALS AND METHODS**

**Diversification experiment**

A single clone of P. fluorescens SBW25 was used to found experimental lines at each combination of resource supply and predation. A gradient of resource supply was constructed by two-fold dilution of KB nutrients (glycerol and proteose peptone) in M9 salt solution (NH₄Cl, 1 g·l⁻¹; Na₂HPO₄, 6 g·l⁻¹; KH₂PO₄, 3 g·l⁻¹; NaCl, 0.5 g·l⁻¹) eight times, ranging from 1× the standard concentration (proteose peptone, 20 g·l⁻¹; glycerol, 12 g·l⁻¹) down to 0.008× (proteose peptone, 0.16 g·l⁻¹; glycerol, 0.09 g·l⁻¹). This follows the procedure described by Kassen et al. (2000) except that we excluded concentrations of 2× the standard
and higher because we found that *T. thermophila* does not survive at these levels, presumably because of some physiological stress imposed by the extremely high concentrations of proteose peptone and glycerol. Even so, this range of concentrations generates considerable variation in bacterial growth \((F_{7,16} = 15.54, P < 0.001)\), ranging from \(~1 \times 10^8\) CFU·ml\(^{-1}\) at the lowest to \(~3 \times 10^9\) CFU·ml\(^{-1}\) at the highest concentration. The experiment was carried out in static 28-ml glass vials containing 6 ml of liquid media. Each culture was inoculated with \(~10^3\) cells of the ancestral clone and then incubated at 28°C for 6 days. Three replicate cultures were maintained at each level of resource supply both with and without *T. thermophila*, of which approximately \(~10^3\) cells were introduced at the start of the experiment.

At the end of the experiment, all cultures were vortexed for 45 s before dilution and spreading on KB agar plates. The frequencies of different morphotypes were then estimated by counting the number of viable colonies. Visibly distinct morphotypes were propagated in KB and plated again to ensure that differences were heritable. The frequency data were analysed using a generalized linear model in R (version 2.2.0) with a quasi-binomial error structure to account for over-dispersion, with predation, resource supply, and their interaction as factors. Diversity was measured as the complement of Simpson’s index, \(1 - \lambda\), where \(\lambda = \Sigma p_i^2\) and \(p_i\) is the frequency of each morphotype. This measures the probability that two randomly selected colonies are different. Diversity scores were normalized by Box-Cox transformation and population densities were log-transformed. We also tested for variation in richness – the number of different morphotypes in a given culture – using analysis of variance, with resource supply and predation as factors. Furthermore, we tested for an underlying association between richness and WS frequency using analysis of covariance, with richness as the response variable, predation as a factor, and square-root transformed WS frequency as a covariate, with the interaction term testing for a difference in the slope of this relationship between predated and non-predated cultures.

**Growth assays in pure and mixed cultures**

We tested the effect of competition among morphotypes by comparing the frequencies of the two principal morphs (SM and WS) in pure and mixed culture at three different resource concentrations: \(1 \times\), \(0.125 \times\), and \(0.008 \times\). In mixed cultures WS and SM were grown in the same vial, while in pure cultures they were grown in separate vials, so that a pair of pure culture vials may be considered a single pure culture growth assay. This experiment was carried out both with and without predation by *T. thermophila*; independent cultures were sampled every day for 4 days. Microcosms were inoculated at low density with approximately \(~10^3\) cells of either or both of the ancestral SM and a derived WS of the most common subclass from the end of the experiment. Three cultures at each combination of nutrient concentration, predation, and competition were then incubated and destructively sampled as above each day for 4 days. The frequencies of WS and SM at each time point were then estimated from colony counts as above.

To analyse the proportion of WS in each assay, we fitted generalized linear models as above, with competition (pure or mixed culture), resource concentration, predation, and day as explanatory variables in a fully factorial model. The maximal model was then reduced by sequentially removing non-significant interactions using \(F\)-tests (Crawley, 2002). Given that destructively sampled microcosms represent independent data points, these data are not repeated measures in the strict sense. However, because samples were taken
over successive days, we accounted for multi-sample sphericity in testing for day effects by adjusting the degrees of freedom using a Greenhouse-Geisser estimate of epsilon ($\varepsilon$).

Low WS frequency in a given assay may be due either to reduced numbers of WS or to increased numbers of SM. We discriminated between these alternatives by testing the effect of each factor on the log-transformed population densities of SM and WS separately and at each level of resource supply. The proportion of explained variance for each factor was estimated as $\omega^2$ using the following equation:

$$\omega^2 = \frac{\text{SS}_{\text{factor}} - (p - 1)\text{MS}_{\text{residual}}}{\text{MS}_{\text{residual}} + \text{SS}_{\text{total}}},$$

where $p$ is the number of levels of a given factor. In some pure cultures (36/144), diversification was detected over the course of the assay. However, there was no significant reduction in the predictive power or qualitative conclusions of models when these cases were excluded and we found no points with high influence (maximum Cook's $D_i = 0.1$).

**RESULTS**

Frequency of resistant individuals varies with resource supply

The proportion of cells represented by WS relative to SM was increased under predation ($F_{1,32} = 64.40$, $P < 0.001$; Figs. 1a, b), but the effect varied with resource supply (predation $\times$ resource supply interaction: $F_{7,32} = 3.45$, $P < 0.01$), and was greatest at intermediate levels. Predation by *T. thermophila* also reduced the population density in each culture ($F_{1,32} = 111.79$, $P < 0.0001$), decreasing the total number of cells by a factor of $\sim 9.7$ on average. While the proportion of the SM population removed by predation appears to be independent of resource supply [effect of predation $\times$ resource supply interaction on log(SM population density): $F_{7,32} = 1.04$, $P = 0.42$], the proportional reduction in WS population density due to predation was greatest at higher concentrations (predation $\times$ resource supply interaction: $F_{7,32} = 3.16$, $P = 0.01$), where WS was numerically dominant. In total, we observed four different types of WS and two types of SM, as well as a third morphological class that occupies the bottom of the vial (fuzzy spreader: FS), although this was only found in 2 of 48 cultures and at very low frequency ($< 0.01$).

Diversity reflects changes in morphotype frequencies

Increased WS frequency under predation led to an increase in diversity ($F_{1,32} = 39.21$, $P < 0.0001$; Fig. 1c). The strength of this effect varied with resource supply (predation $\times$ resource supply interaction: $F_{7,32} = 3.32$, $P < 0.01$), peaking at intermediate levels where the increase in WS frequency was greatest. This is due to the fact that diversity tended to be greater within WS than SM populations (WS: mean = 0.436, s.d. = 0.151; SM: mean = 0.001, s.d. = 0.004). In turn, because $1 - \lambda$ is maximized when the frequencies of morphotypes both within and between the principal classes are approximately equal, diversity was greatest when WS attained a frequency of 0.65–0.75, not when WS and SM were equally abundant.

Diversity can also be interpreted in terms of the number of different morphotypes in each culture, which is analogous to species richness. Richness was not affected by predation overall ($F_{1,32} = 0.25$, $P = 0.62$), but did vary with resource supply ($F_{7,32} = 6.61$, $P < 0.0001$), tending to be higher in high-resource treatments. This probably reflects an underlying relationship between richness and the frequency of wrinkly spreaders, with the number of
Fig. 1. Frequency of the principal morphotypes following diversification in static microcosms at different levels of resource supply both without (a) and with (b) predation by *T. thermophila*. Bars show means ± standard errors for three populations. Also shown are the resulting patterns of diversity (c) in the absence (Tt−) and presence (Tt+) of *T. thermophila*. Points show means ± standard errors for three populations.
morphotypes detected being lower for cultures with a smaller proportion of WS (regression against square-root transformed WS frequency: $r^2 = 0.30$, $n = 48$, $P < 0.0001$). The slope of this relationship was unaffected by the presence of *T. thermophila* (predation $\times$ WS frequency interaction: $F_{1,44} = 1.18$, $P = 0.28$). Thus, the effect of predation on diversity largely reflects changes in the frequencies of different morphotypes, rather than changes in the number of morphotypes at the end of diversification.

The interaction between competition and predation varies with resource supply

In pure culture assays, the frequency of WS relative to SM increased with resource supply, both in the presence and absence of predators (Fig. 2). In some treatments, WS frequency was reduced by competition with SM in mixed cultures, but this effect depended upon both resource supply ($F_{2,105} = 13.93$, $P < 0.0001$) and predation ($F_{1,105} = 8.76$, $P < 0.005$). Specifically, in low-resource environments, WS frequency was reduced by competition with SM both with and without predation by *T. thermophila* ($F_{1,33} = 32.32$, $P < 0.001$; Fig. 2), and there was no interaction between competition and predation ($F_{1,32} = 0.44$, $P = 0.51$). At

![Fig. 2. Proportion of cells represented by WS relative to SM in pure and mixed culture assays at three levels of resource supply both in the absence (a) and presence (b) of *T. thermophila*. Bars show means ± standard errors for 12 populations.](image-url)
intermediate resource supply, the effect of competition depended upon the presence of the predator (competition × predation interaction: $F_{1,31} = 5.44, P = 0.02$), and WS frequency was decreased by competition only in the absence of *T. thermophila* (no predation: $F_{1,20} = 5.72, P = 0.02$, Fig. 2a; predation: $F_{1,18} = 0.06, P = 0.81$, Fig. 2b). In high-resource environments, where WS was numerically dominant, there was no significant main effect of competition ($F_{1,32} = 1.72, P = 0.19$) or predation ($F_{1,32} = 0.93, P = 0.34$) on WS frequency.

Is the effect of competition in mixed cultures due to changes in the abundance of WS, SM or both? Overall, competition led to reduced WS population density ($F_{1,121} = 30.06, P < 0.0001$), while SM population density was unaffected ($F_{1,128} = 0.28, P = 0.60$). The reduction in WS density was greatest in low-resource environments (29% variance explained, compared with 10% and 5% at intermediate and high levels respectively). Having found that the effect of competition on WS frequency was nullified by predation at intermediate resource supply (Fig. 2), we also wished to determine if this was due to increased numbers of WS under predation, or to reduced numbers of SM. We found that the reduction in WS population density due to competition was independent of predation in these treatments (competition × predation interaction: $F_{1,28} = 0.82, P = 0.37$), but that SM population density was reduced under predation in mixed cultures ($F_{1,35} = 36.26, P < 0.0001$; Figs. 3e, k). Therefore, competition generally reduces WS frequency because

**Fig. 3.** Population density of SM smooth (grey circles) and WS wrinkly spreader (open squares) morphotypes over 4 days in pure and mixed culture at three different levels of resource supply. Bars show means ± standard errors for three populations.
of reduced growth and survival in mixed cultures, but this effect is countered by predation in some treatments, which leads to decreased growth of the competing SM population.

**DISCUSSION**

The overall effect of predation was an increase in diversity due to increased frequency of the biofilm-forming WS phenotype emerging during adaptive diversification. Interestingly, the magnitude of this effect varied across a gradient of nutrient supply, being smallest at the two extremes and largest at intermediate levels. In these treatments, a reduction of the competing SM population under predation was combined with sufficient resource supply to maintain a substantial biofilm, leading to increased WS frequency and therefore higher diversity. Thus, predation increased the range of concentrations over which high diversity was supported.

These observations are consistent with our knowledge of the evolutionary ecology of *P. fluorescens* in laboratory microcosms, and also with previous work showing that selection for resource exploitation is strongest when resources are limited (Leibold, 1996; Bohannan and Lenski, 2000). In resource-poor conditions, nutrient limitation prevented the formation of the energetically costly WS biofilm, and the pelagic SM population was competitively dominant both with and without predation. As nutrient supply increased, the benefits of growth at the air–liquid interface outweighed the energetic costs of biofilm formation and WS emerged through resource competition in the absence of predation. In the presence of predators, WS enjoyed a further advantage because growth in the biofilm also conferred predator resistance. The competing SM population was more susceptible to predation, as evidenced by the reduction in SM density under predation at intermediate nutrient supply (compare Figs. 3b and e with h and k).

At the highest levels of resource supply, WS were numerically dominant even in the absence of predators, and removal of a proportion of the broth-living SM population in the predator treatments led to a relatively small change in WS frequency. This effect was compounded by the fact that predators also removed a greater proportion of WS cells when they were more abundant. This is consistent with the previous finding that the advantage of predator resistance in the biofilm is reduced when WS are in the majority (Meyer and Kassen, 2007). Thus, contrary to our expectation that selection for predator resistance would be more important at high resource concentrations, we found that it had a relatively small effect because the same phenotypes that are resistant to predators are also preponderant in their absence.

Diversity is often seen to display a unimodal relationship with nutrient supply (Tilman, 1982; Rosenzweig, 1995). This is also true for *P. fluorescens*, as diversity has been shown to drop at concentrations above the highest level used here (Kassen et al., 2000). Thus, our results do not contradict the notion of a ‘paradox of enrichment’ (Rosenzweig, 1971) for this system. However, we do find that the range of concentrations that support high diversity is extended under predation, and therefore suggest that the characteristic ‘hump-shaped’ relationship between diversity and resource supply may be broadened by predation.

In a recent study, Benmayor et al. (2008) showed that the presence of parasitic bacteriophage in *P. fluorescens* cultures can lead to an analogous increase in diversity, because of selection for the phage-resistant ‘fuzzy spreader’ phenotype that occupies the bottom of the vial. However, Benmayor et al. (2008) found no significant variation in FS frequency with either resource concentration or the frequency of disturbance. These
apparently contrasting outcomes may reflect ecological differences between growth in the biofilm and at the bottom of the vial. The productivity of the biofilm niche relative to the broth phase varied with resource supply as described above. However, the relative productivity of growth at the bottom of the vial was probably limited by the lack of oxygen at this niche, and thus was effectively independent of changes in nutrient concentration. This would mean that the selective benefit of phage resistance for FS is fixed across a gradient of nutrient supply. It is interesting that both parasites (Benmayor et al., 2008) and predators (present study) can generate similar patterns of diversity, although the underlying mechanisms responsible may vary.

In our experiment, predation modulated the frequencies of phenotypes that also occur during diversification under resource competition alone. However, predators can generate diversification of _P. fluorescens_ to distinct morphotypes independently of resource competition. This has been demonstrated by diversification under predation in minimal growth medium that does not support diversity in the absence of _T. thermophila_ (Meyer and Kassen, 2007). Furthermore, although we looked at the effect of predation in spatially heterogeneous microcosms, spatial structure is not necessary for predation to promote diversification (Levin et al., 1977), as demonstrated by recent experimental work with _P. fluorescens_ (Gallet et al., 2007). All that is required is that there is a trade-off between resistance and competitive fitness or growth rate (Levin et al., 1977; Chase et al., 2002). Our results support the notion that the effect of such a trade-off varies with resource supply (Holt et al., 1994; Bohannan and Lenski, 2000). Since the diversifying selection resulting from predation depends upon the relative fitness of resistant and susceptible phenotypes, a single phenotype may dominate at the extremes. In general, diversification may be restricted if there is strong selection for a single resistance strategy (Buckling and Rainey, 2002) or for resource exploitation, such as when environmental nutrient supply is extremely low.

In the present experiment and several others with _P. fluorescens_ (e.g. Rainey and Travisano, 1998; Buckling and Rainey, 2002; Kassen et al., 2004), diversity is measured using the frequencies of different morphotypes. If there were no variation within morphotypic classes, then overall diversity would be maximized when SM and WS are equally abundant. However, we found greater diversity within WS than SM populations, and therefore diversity was actually greatest when WS was numerically dominant. High morphological diversity within WS populations is probably due to the differences between growth in a biofilm and in the liquid phase. Biofilms are structurally complex (Spiers and Rainey, 2005) and are generally more diverse and dynamic than pelagic communities (Sutherland, 2001; Boles et al., 2004). Thus, although diversity was greatest in high-resource environments, the ratio of WS to SM was most even at intermediate resource concentrations under predation. This is in line with the notion that co-existence of resistant and sensitive phenotypes is most likely at intermediate levels of nutrient supply, because one type is limited by predation and the other by competition for resources (Abrams, 1993; Holt et al., 1994; Leibold, 1996).

Recent experimental work has highlighted the importance of predators in evolution (Rundle et al., 2003; Meyer et al., 2006; Nosil and Crespi, 2006). Our results support the idea that predation can promote diversification due to the co-existence of sensitive and resistant prey, both in general (Holt et al., 1994; Chase et al., 2002) and for this species in particular (Gallet et al., 2007; Meyer and Kassen, 2007). More importantly, the role of predators during adaptive diversification appears to vary with resource availability.
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