Killer prey: Temperature reverses future bacterial predation

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Abstract

Ecological variation influences the character of many biotic interactions, but examples of predator-prey reversal mediated by abiotic context are few. We show that the temperature at which prey grow before interacting with a predatory bacterial species can determine the very direction of predation, reversing the identities of predator and prey. When lawns of *Pseudomonas fluorescens* were reared at 32 °C before release of the generalist predator *Myxococcus xanthus, M. xanthus* extensively killed *P. fluorescens*. However, when *M. xanthus* was released onto lawns of *P. fluorescens* reared at 22 °C, *P. fluorescens* was the predator, slaughtering *M. xanthus* to extinction and growing on its remains. Cooler-reared *P. fluorescens* killed *M. xanthus* by secreting diffusible molecules that, while lethal to *M. xanthus,* were benign toward most of several other diverse bacterial species examined. Our results suggest that the sign of predator-prey interactions – and lethal microbial antagonisms more broadly – may often change across abiotic gradients in natural microbial communities, with important ecological and evolutionary implications. They also suggest that a larger proportion of microbial warfare results in predation – the killing and consumption of organisms – than is generally recognized.

Significance Statement

The roles of abiotic ecology in shaping the lethal warfare that pervades microbial communities are only beginning to be understood. We find that variation in a single physical property of a microbial habitat - temperature - reverses the direction of both killing and predation in an inter-specific bacterial interaction and does so by future-acting rather than contemporaneous effects. These outcomes suggest that the identities of killers and victims in microbial warfare - and of predators and prey when victims are consumed by their killers - might often reverse across ecological gradients. Additionally, finding that a known prey species can kill and consume a known predator in an ecologically-contingent manner suggests that much of microbial warfare culminates in predation.

Main Text

Introduction

Ecological context strongly shapes the intensity and character of many biotic interactions (1, 2), including between predators and prey (3). Some ecological factors influence predator-prey interactions contemporaneously. For example, water temperature immediately influences the timing and duration of predator attacks in some fish (4), background *vs.* prey coloration often determines prey detectability (5), and snow depth modulates the efficiency of wolf predation (6). Most such examples illustrate how ecological context modulates the effectiveness of predation in a unidirectional predator-prey relationship. However, at least one study has found that abiotic ecology can even reverse the predominant direction of mutual predation; specifically, the majority-direction of predation events between two amphipod species has been shown to reverse as a function of salinity (7).

Ecological effects on predator-prey interactions can also be delayed. For example, maternal exposure to predation risk at one time can influence the degree of anti-predator behavior displayed by offspring at a later time (8). Investigating how predator-prey interactions change as a function of ecological context, whether contemporaneous or historical, is necessary for understanding how those interactions evolve in spatially and temporally heterogeneous habitats.

Predation pervades the microbial world (9) as well as animal communities, impacting microbial-community composition and network structure and thereby influencing nutrient-cycling dynamics and important features of macro-organism biology. Behavioral modes of microbial predation vary greatly. Many eukaryotic predators of bacteria such as nematodes and many protists ingest whole prey cells (10, 11), while predatory bacteria are too small to do so. In a virus-like life-cycle, cells of the specialist bacterial predator *Bdellovibrio bacteriovorus* physically attach to a prey cell before invading it and reproducing inside (12). Other bacteria kill prey without attachment using diffusible secretions (13, 14). In addition to using diffusible killing agents, the generalist bacterial predator *Myxococcus xanthus* employs highly effective predatory weapons that depend on contact with prey cells (15, 16). Perhaps best known for its formation of fruiting bodies in response to starvation (17), *M. xanthus* can prey on a very broad range of other microbes, including both Gram- and Gram+ bacteria (18) and some fungi (19). *M. xanthus* uses two synergistic motility systems to forage for prey across variable solid surfaces (20).

Heterogeneity in many abiotic factors such as temperature, prey nutrition, pH, oxygen availability, salinity, and surface or fluid viscosity is known or likely to influence microbial predator-prey interactions (21–26), and microbial antagonisms more broadly (27–30). For example, predators and prey may be differentially sensitive to pH gradients (25, 31) and temperature influences many bacterial traits relevant to predation, including cell-division rates and motility

behavior (32, 33). Temperature also impacts secondary metabolite production (34, 35) and Type-VI secretion (36, 37), traits associated with a broad range of microbial antagonisms often not described as predatory, and can modulate the intensity of such antagonisms (38).

Motivated by observations suggesting that *M. xanthus* predation could differ greatly on prey reared on a room-temperature bench *vs.* in a warmed incubator, we investigated how variation in the temperature at which bacterial prey grow prior to predator attack might alter the predation risk of prey. Using a phylogenetically diverse panel of bacteria previously demonstrated to fuel predatory growth by *M. xanthus* (18, 39), we initially quantified the ability of *M. xanthus* to swarm through prey lawns reared at three temperatures (12, 22 or 32 °C). Upon observing that *Pseudomonas fluorescens* eliminated *M. xanthus* swarming when the former had been previously reared at 22 °C but not at 32 °C, we quantified *M. xanthus* viable-population size after interaction with *P. fluorescens* pre-grown at the same three temperatures from several initial densities. Interaction with *P. fluorescens* grown at 22 °C resulted in *M. xanthus* extinction. We then tested whether the killing factors produced by this sometime-prey species are cell-bound or diffusible secretions and whether they are proteinaceous or not (or require polypeptides to function) by testing for functional sensitivity to 95 °C heat exposure. We further asked whether *P. fluorescens* functions as a predator after killing *M. xanthus* by testing whether nutrients from killed *M. xanthus* fuel *P. fluorescens* growth. Finally, we tested whether *P. fluorescens* secretions lethal to *M. xanthus* also kill a diverse panel of other potential prey species.

Results

M. xanthus swarming on P. fluorescens lawns depends on prey-rearing temperature

We first tested whether varying the temperature at which potential prey bacteria grow prior to interacting with *M. xanthus* would impact the rate at which *M. xanthus* populations swarm through prey lawns at a single temperature after predator release. Previously, the rate at which *M. xanthus* swarms through prey lawns on an agar surface has been shown to correlate with several other measures of predatory performance (39). We performed these assays on M9cas agar containing 0.3% casitone, which *M. xanthus* can use to fuel growth in the absence of prey. However, if prey are first grown in lawns on M9cas before *M. xanthus* is added, the prey sequester nutrients from the casitone into prey-cell biomass and *M. xanthus* cells can potentially fuel growth from prey cells by killing and consuming them. They might also fuel some growth from any residual casitone nutrients not incorporated into cells by prey.

Lawns of six diverse bacterial species known to serve as prey to *M. xanthus* (*Arthrobacter globiformis, Bacillus bataviensis, Escherichia coli, Micrococcus luteus, Pseudomonas fluorescens* and *Rhizobium vitis* (39)) were reared on M9cas agar for 22 h at 12, 22 or 32 °C and then all

incubated at room temperature for 2 h prior to release of *M. xanthus*. Aliquots of three *M. xanthus* strains (GJV1, A75, and Serengeti 01; 20 µl at ~10^{10} cells/ml) were spotted at the centers of the prey lawns and allowed to grow and swarm outward to their ability for seven days at 32 °C before swarm diameters were measured.

Across the entire dataset, swarm sizes were impacted by prey presence, prey identity, *M. xanthus* genotype and the rearing temperature of prey, as well as interactions among these factors ($F_{62,171}$ = 38.08, $p < 0.01$, Supplementary table S1A). Swarming rates in the absence of any prey were faster than swarming across prey lawns (Fig. 1, Tukey-adjusted contrasts averaged over interaction terms, all $p < 0.01$).

Prey-rearing temperature had no effect on the swarming rates of any *M. xanthus* genotype for five prey species but strongly impacted swarming of all *M. xanthus* genotypes on lawns of *P. fluorescens* (Tukey-adjusted contrasts for each predator-prey combination, Table S1B). All three *M. xanthus* genotypes swarmed effectively when *P. fluorescens* had grown at 32 °C, swarmed relatively poorly when the prey were grown at 12 °C and did not grow detectably at all when the prey were grown at 22 °C. Thus, the temperature at which *P. fluorescens* prey lawns grew prior to encountering a bacterial predator strongly determined the predator's ability to swarm through those lawns.

M. xanthus kills P. fluorescens reared at 32 °C on M9cas agar

Although *M. xanthus* was previously shown to utilize *P. fluorescens* as prey to fuel population growth (18, 39) while substantially reducing *P. fluorescens* population size (18), the composition of agar medium used in the earlier experiments differed from the M9cas agar used in the Fig. 1 swarming assays. Because both species had been reared at 32 °C before being mixed in the earlier experiments, we sought to confirm our expectation that GJV1 kills *P. fluorescens* reared overnight at 32 °C on M9cas agar. Indeed, when GJV1 was distributed across the surface of circular *P. fluorescens* lawns reared at 32 °C on M9cas, the *P. fluorescens* populations were reduced by ~90% relative to control populations lacking *M. xanthus* after four days of *M. xanthus* growth (Fig. S1). From this result, we infer that, in the experiment reported in Fig. 1, *M. xanthus* extensively killed *P. fluorescens* within the areas covered by *M. xanthus* swarms expanding through 32 °C-reared *P. fluorescens* lawns.

P. fluorescens reared at lower temperatures kills M. xanthus

The observed inhibition of *M. xanthus* growth by lawns of *P. fluorescens* reared at 22 °C (Fig. 1) might be caused by either a non-lethal mechanism that merely prevents (or greatly slows) *M. xanthus* cell division, or by killing. To test between these hypotheses, 20-µl aliquots containing ~2 x 10^8 cells of *M. xanthus* strain DK3470 were spotted onto lawns of *P. fluorescens* previously grown

overnight at 12, 22 or 32 °C. *M. xanthus* population size was then estimated ~30 min and seven days after *M. xanthus* inoculation. DK3470 was used for this assay rather than GJV1 or the natural isolates because DK3470 disperses more readily due a mutation that prevents production of adhesive extracellular matrix. As part of the same experiment, we also tested whether the initial population size of *P. fluorescens* inoculated to initiate growth prior to meeting *M. xanthus* might impact any effect of *P. fluorescens* on *M. xanthus* population size after addition of the predator. To do so, *P. fluorescens* growth was initiated from culture aliquots at three inoculum sizes (~10^5, 10^6 and 10^7 cells/flask) the day prior to addition of *M. xanthus*.

Consistent with the swarming results shown in Fig. 1, lawns of *P. fluorescens* reared at 32 °C supported the largest *M. xanthus* populations after a week of incubation (Fig. 2, Tukey-adjusted contrasts averaged over interaction terms, all $p < 0.01$). Prey inoculum size had no impact on *M. xanthus* population size when *P. fluorescens* had been reared at 32 °C.

In contrast, and also consistent with the Fig. 1 swarming results, *P. fluorescens* reared at 22 °C prior to meeting *M. xanthus* did not merely limit predator growth, but slaughtered *M. xanthus* to extinction, irrespective of initial prey population size (Fig. 2, Table S2). No viable *M. xanthus* cells were observed at the limit of detection in any of the treatments in which *P. fluorescens* was reared at 22 °C.

Unlike in the two higher-temperature rearing treatments, prey inoculum size greatly impacted *M. xanthus* population size for *P. fluorescens* reared at 12 °C (Fig. 2, rearing-temperature x prey-inoculum size interaction term $F_{6,59} = 42.1$, $p < 0.01$, Table S2). Like *P. fluorescens* reared at 22 °C, some cultures reared at 12 °C also killed *M. xanthus* to extinction, but did so only when *P. fluorescens* was inoculated at the highest inoculum size. When *P. fluorescens* was inoculated at the two lower inoculum sizes prior to growth at 12 °C, *M. xanthus* grew to population sizes similar to those achieved on *P. fluorescens* reared at 32 °C. Killing of *M. xanthus* by *P. fluorescens* grown at 22 °C (from any inoculum size) or 12 °C (from high inoculum size) occurred rapidly, being observed already after only 30 min after inoculation of *M. xanthus* (Fig. S2).

Together, the above results demonstrate both that i) variation in a single abiotic parameter can determine the direction of killing between a predatory bacterial species and one of its prey species and ii) such abiotic determination of killing direction can be exerted remotely in time, with the temperature at which *P. fluorescens* grows prior to interaction with *M. xanthus* determining which species kills the other.

Killing of M. xanthus by 12 °C-grown P. fluorescens appears contingent on post-growth population density

We hypothesized that, in the Fig. 2 experiment with DK3470, population densities of *P. fluorescens* after overnight growth might have mediated some effects of *P. fluorescens* on DK3470 growth or

death, whether partially or fully. To examine this hypothesis, we cultivated *P. fluorescens* from the same three initial population sizes and at the same three temperatures as in the Fig. 2 experiment and then assessed population sizes after 22 h of growth at the three temperatures and subsequent incubation at room temperature for 2 h.

Post-growth population size of *P. fluorescens* (*i.e. N* after 24 h total incubation) was independent of initial population size after growth at 32 °C (R^2 = 0.24, $F_{1,7}$ = 2.25, p = 0.18, Fig. S3) but correlated positively with initial population size after growth at 12 and 22 °C (R^2 = 0.996, $F_{1,7}$ = 1709 for 12 °C; R^2 = 0.79, $F_{1,7}$ = 26.58 for 22 °C, both p < 0.01), with the strongest relationship observed after growth at 12 °C. The sizes of *P. fluorescens* populations grown at 32 °C (from any initial population size) did not differ from those of populations grown at 22 °C from the highest inoculum size. Because all 22 °C-grown populations killed *M. xanthus* but no 32 °C-grown populations did so, our results do not indicate that *P. fluorescens* post-growth population density has any effect on the efficacy with which 22 °C-grown *P. fluorescens* kills *M. xanthus,* at least over the range observed here.

In contrast, because post-growth population sizes of 12 °C-grown *P. fluorescens* correlated strongly with initial population sizes and because only those populations initiated at the highest inoculum size killed *M. xanthus* (Fig. 2), killing of *M. xanthus* by 12 °C-grown *P. fluorescens* appears to depend positively on post-growth population density.

The lethal compound produced by 22 °C-grown P. fluorescens is a non-proteinaceous, diffusive secretion

We tested whether the molecules lethal to *M. xanthus* produced by 22 °C-grown *P. fluorescens* are cell-bound or diffusive and whether they are proteinaceous. *P. fluorescens* was grown into lawns overnight at 12, 22 and 32 °C from an initial population size of ~10^7 cells. The resulting lawns were suspended in buffer solution using sterile glass beads and sterile supernatant was extracted after centrifugation and filtration. Supernatant samples were then split; one was heated at 95 °C to test for heat inactivation of protein function while the other was kept at room temperature. Centrifuged pellets of *M. xanthus* strain DK3470 were then resuspended with the supernatant samples and the resulting suspensions incubated for 6 hours before dilution plating to determine viable population sizes.

Supernatant from 32 °C-grown *P. fluorescens,* whether heated at 95 °C or not, had no effect on DK3470 population size ($F_{11,24}$ = 441.2, p < 0.01, Tukey-adjusted contrasts t_{24} = 0.897, p = 0.38 for heated treatment and t_{24} = -1.567, p = 0.13 for non-heated treatment, Fig. 3). Supernatant from 12 °C-grown populations slightly reduced *M. xanthus* population size, at least when heated (t_{24} = 2.953, p < 0.01 for heated treatment and t_{24} = 0.92, p = 0.37 for non-heated treatment). Most strikingly, supernatant from 22 °C-grown *P. fluorescens* killed *M. xanthus* populations completely,

both when heated and not (t_{24} = 39.33 and 38.33 for heated and non-heated treatments, respectively; $p < 0.01$ for both). Because cell-free supernatant from the 22 °C-grown populations effectively killed the predator and 95 °C heat did not impair killing, we infer that the killing agent is a non-proteinaceous, diffusive secretion.

Killed M. xanthus fuels P. fluorescens population growth

We asked whether the interaction between 22 °C-reared *P. fluorescens* and *M. xanthus* involves only killing or rather predation, which involves both killing and consumption of the organisms killed. To do so, we tested whether *P. fluorescens* can grow on nutrients derived from *M. xanthus* cells killed by 22 °C-reared *P. fluorescens*.

Cell-free supernatant from suspensions of 22 °C-reared *P. fluorescens* lawns was prepared as in the previous experiments and then used to test for *P. fluorescens* growth in four treatments. Half of the *P. fluorescens* supernatant was used to resuspend centrifuged pellets of *M. xanthus* strain DK3470; those resuspensions were then incubated for 6 h to kill *M. xanthus.* Samples from these resuspensions plated on CTT agar after the 6 h incubation period showed no evidence of *M. xanthus* growth five days after plating, confirming that the *P. fluorescens* supernatant killed *M. xanthus* to extinction, as in the previous experiments. No *M. xanthus* cells were added to the other half of the *P. fluorescens* supernatant.

We then inoculated *P. fluorescens* at two initial densities (~10^6 and ~10^8 CFU/ml) into the *P. fluorescens* supernatant to which *M. xanthus* cells had either been added or not and monitored subsequent population densities. While *P. fluorescens* unsurprisingly grew to some degree on nutrients present in its own supernatant alone (Fig. 4) (40), from both initial densities it grew to much higher densities in the supernatant containing killed *M. xanthus* (49- and 22- fold higher for the low and high initial *P. fluorescens*-density treatments, respectively; $F_{1,8}$ = 43.11, $p < 0.01$, Tukey-adjusted contrasts t_8 = -8.84, $p < 0.01$, Fig. 4). From these results, we infer that *P. fluorescens* reared at 22 °C in our earlier experiments underwent significant growth fueled by molecules derived from *M. xanthus* cells that they killed. By growing on the remains of *M. xanthus* populations that they killed, *P. fluorescens* cells grown at 22 °C are predators of *M. xanthus*.

P. fluorescens supernatant lethal to M. xanthus harms a minority of diverse tested species

We began investigating the killing range of the compound(s) lethal to *M. xanthus* produced by 22 °C-grown *P. fluorescens* by exposing cells from seven other bacterial species to the *M. xanthus*-lethal supernatant. We tested both Gram+ and Gram- species, including cells of *P. fluorescens* itself as controls. Although *M. xanthus* was again killed effectively in these assays (Fig. 5; $F_{7,32}$ = 102.89, $p < 0.01$, Tukey contrasts on supernatant *vs* control marginal means t_{32} = 29.45 $p < 0.001$), only two of the other examined species were significantly harmed by *P. fluorescens* supernatant

(Fig. 5). *B. bataviensis* and *M. luteus* populations exposed to the supernatant were reduced to less than 10% and 50% of their corresponding control populations, respectively (t_{32} = 5.7 p < 0.001 and t_{32} = 2.69 p < 0.05, respectively), whereas the other species were not significantly affected (Tukey contrasts on supernatant *vs* control marginal means, all p > 0.05).

Discussion

The animal world features numerous examples of predation role reversal (41–43) and mutual predation (44–46), and at least some are determined by ecological context (7). In the microbial world, ecological factors have been shown to greatly impact predation efficiency but, to our knowledge, without reversing the direction of predation. We have presented an extreme example of the importance of the abiotic environment; changing a single physical variable in the growth conditions of one species reverses the character of its subsequent interaction with another. Specifically, changing the temperature at which a bacterial species grows prior to interspecific interaction flips hierarchy in a food chain with the sometime prey species *P. fluorescens* becoming a predator of the often-predacious species *M. xanthus*. Reversed killing of *M. xanthus* by *P. fluorescens* is mediated by extremely effective non-proteinaceous diffusible molecules. However, *P. fluorescens* supernatant containing the killing compound(s) harmed only three of seven examined bacterial species (including *M. xanthus*) and did so in a phylogenetically idiosyncratic manner.

Mechanism and range of killing

Thoroughly understanding the selective forces shaping production of an antimicrobial compound by bacteria residing in species-rich communities is a complex challenge. This requires quantification of both production costs and of how the compound's antagonistic effects on producers' diverse neighbors collectively impact producer fitness. Such fitness effects include benefits of harming or killing neighbors derived both from securing competed-for resources and from nutrients obtained directly from prey cells. Such thorough understanding would also require characterization of compound features such as energy requirements for its synthesis, how its production is regulated, whether it is cell-bound or diffusible, and, if diffusible, its spatial diffusion ranges across producer habitats and the temporal durability of the compound's antagonistic effects under relevant ecological conditions.

Several of our results are first steps toward understanding the evolutionary character of the compound produced by cool-reared *P. fluorescens* that is lethal to *M. xanthus*. As previously mentioned, it is a diffusible secretion the production of which is regulated by temperature. It is

functionally robust after having been heated to 95 °C, indicating that it is not a protein, and a modified drop-collapse assay (as in (47)) performed with supernatant from our experiments suggests that the lethal compound is a biosurfactant. In our experiments, the compound was produced prior to interaction with *M. xanthus* and thus mediated opportunistic predation. The negative effects of *P. fluorescens* supernatant on *B. bataviensis* and *M. luteus* populations (Fig. 5) may be caused by the same compound(s) that exterminated *M. xanthus*. If this were the case, our results would indicate that its mechanism of antagonism is unrelated to Gram-type cell-wall specificities; both Gram+ and Gram- species were harmed by the supernatant and other species of both Gram types were unharmed. Thus, if the same compound(s) kills the three sensitive species, the phylogenetic distribution of its killing range appears to be highly idiosyncratic.

Apparent density-dependent killing

Some known predators such as *M. xanthus* and *Streptomyces* spp. secrete diffusible toxins capable of killing many prey cells at a distance (48). Similarly, temperature-dependent killing of *M. xanthus* by *P. fluorescens* is carried out by a diffusible secretion. Diffusible secretions are known to mediate density-dependent behaviors in bacteria (49). It appears that killing of *M. xanthus* by *P. fluorescens* is also density dependent in at least some ecological contexts.

Among *P. fluorescens* populations reared at 12 °C, only populations initiated from the highest starting density in our experiments – which in turn reached the highest final density before meeting *M. xanthus* – killed *M. xanthus* (Figs. 2 and S3). This suggests that, at low temperatures, a threshold *P. fluorescens* density needs to be reached for secretions lethal to *M. xanthus* to be produced in quantities sufficient to kill extensively. This effect might result simply from a lethal compound increasing linearly with local population size; or compound production might be regulated by a form of quorum sensing. However, high density alone is insufficient to allow predation of *M. xanthus* by *P. fluorescens*; *P. fluorescens* populations initiated at the same density and reaching the same final density were lethal after growth at 22 °C but not at 32 °C (Figs. 2 and S3). These results suggest that the efficiency of various forms of microbial killing and predation mediated by diffusible molecules may be positively density dependent, but also suggest that the form or existence of such density-dependence may often depend on ecological context.

Past ecology shapes future interactions

An intriguing feature of our findings is that the observed predator-prey reversal was driven by ecological context prior to, rather than during, predator-prey interaction. In our interaction experiments, only the temperature at which one species (*P. fluorescens*) grew prior to meeting the other (*M. xanthus*) varied across treatments; after *P. fluorescens* growth in isolation, agar plates from all three growth-temperature treatments were brought to the same temperature before the two

species were mixed. Yet differences in *P. fluorescens* biotic environments caused by the differences in growth temperature were not eliminated by the subsequent temperature equalization. *P. fluorescens* reared at 32 °C was extensively killed when exposed to *M. xanthus* (Fig. S1), whereas *P. fluorescens* reared at 22 °C slaughtered *M. xanthus* to extinction (Figs. 2, 3 and S2) and used the released nutrients to fuel its own growth (Fig. 4). The temporal separation of the causative ecological factor and the impacted interaction in our experiments has important implications for our understanding of interaction networks in natural microbial communities. Within such communities, micro-ecological features can change rapidly over time and migration across variable microenvironments can be extensive. Thus, the strength and direction of microbial interactions may often be shaped not only by the abiotic context in which they are taking place, but also by the recent ecological histories of interacting parties.

Myxobacterial predator-prey interactomes

When offered as potential prey, diverse bacterial species vary greatly in the degree to which they fuel *M. xanthus* growth (18, 39) and some exhibit resistance to predation (50). The finding that some bacteria can be predators of myxobacteria rather than their prey should inform future investigations into myxobacterial predator-prey interactomes. Species exhibiting resistance to predation by myxobacteria should also be examined for the ability to kill and consume them. Abiotic ecology, including variables altered by climate change, can be expected to strongly impact the character of predator-prey interactomes between myxobacterial species and other species in both directions of predation and may sometimes determine the very direction of predation across variable natural habitats.

The ecological dependence of microbial predator-prey interactomes and antagonisms more broadly also has applied significance (51–53). The range of ecological conditions under which microbial biocontrol agents can be expected to harm their targets should be investigated in planning their use.

How much microbial warfare results in predation?

P. fluorescens has not often, if ever, been described as a predator, despite its known production of antibiotics (54) and corresponding potential to kill or antagonize other species, including *M. xanthus* (50). Yet because the strain used here can clearly grow on nutrients derived from cells it has killed (Fig. 4), it is indeed a predator under the broad phenomenological definition of ‘predation’ focused on organismal interactions that we adopt. In this definition, ‘predator’ refers to any organism that kills another and then consumes it (55), irrespective of the predator’s evolutionary history of predation. The degree to which an organism’s ancestral lineage has undergone selection favoring nutrient acquisition from predation is not a criterion. This definition has the advantage of focusing

simply on whether a clearly defined interaction occurs, leaving separate the question of that interaction's evolutionary origins, which may often be unclear, especially for microbes.

In light of our results and the ubiquity of intermicrobial killing (29, 56, 57), predation by microbe-killing microbes that have rarely, if ever, previously been labeled as predators is likely to be more common than is generally considered. This includes killing between conspecifics, which occurs pervasively among *M. xanthus* natural isolates (58–60) and is also common in many other species (29). Perhaps most bacterial species engage in predation to some degree. Species frequently labeled as predators likely cluster far along a continuous spectrum of evolutionary adaptedness for predation.

Materials and Methods

Strains

M. xanthus strains GJV1, DK3470, A75, and Serengeti 01 were used in this study. GJV1 is a closely related derivative of the reference strain DK1622 (61), DK3470 is a mutant of DK1622 with a mutation in the *dsp* gene (62), and A75 (63) and Serengeti 01 (64) are natural isolates from Tübingen, Germany, and Serengeti National Park, Tanzania, respectively. Strains of *Arthrobacter globiformis, Bacillus bataviensis, Curtobacterium citreum, Escherichia coli, Micrococcus luteus,* and *Rhizobium vitis* used here are the same as those reported by Morgan and colleagues in 2010 (18). *Pseudomonas fluorescens* strain SBW25 is from (65).

General media and culture conditions

Unless specified otherwise, *M. xanthus* strains were inoculated from frozen stock onto CTT 1.5% agar (1% casitone; 8 mM $MgSO_4$; 10 mM Tris-HCl, pH 7.6; 1 mM KH_2PO_4, 1.5% agar (66)) plates three days prior to transfer of culture samples into 8 ml CTT liquid (identical to CTT agar except lacking agar) in 50 ml flasks. Liquid cultures were typically grown over two days with a dilution transfer after one day. All assays were initiated from log-phase cultures. For other species, samples from frozen stocks were inoculated directly into LB liquid. Unless specified otherwise, liquid cultures were incubated at 32 °C with shaking at 300 rpm and plate cultures were incubated at 32 °C, 90% rH. Cell densities of bacterial populations were estimated with a TECAN Genios™ plate reader. Prior to resuspension to initiate assays, cultures were centrifuged at 4472 x *g*, 15 min. Unless specified otherwise, all bacterial cultures were resuspended in M9 medium (1×M9 salts: 22 mM KH_2PO_4, 18.7 mM NH_4Cl, 8.6 mM NaCl supplemented with 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$) and experiments were run on M9cas agar (M9 medium supplemented with 0.3% casitone and 1.2% agar).

Swarming rates

25-ml aliquots of M9cas agar were poured into 9-cm petri plates and allowed to cool and solidify in a laminar-flow hood without lids for 20 min, after which they were capped and stored overnight at room temperature.

Centrifuged prey cultures were resuspended to a predicted density of ~5 x 10^6 cells/ml in M9 medium. From each resuspended culture (and one control containing only M9), 600 µl aliquots were placed on M9cas-agar plates and distributed evenly with a sterile metal triangle. Plates were then left open without lids in a laminar-flow hood for 60 min. Four plates for each prey species were then incubated at each of three temperatures (12, 22, and 32 °C) for 22 hours. Incubator windows were covered to prevent light penetration. After incubation, the plates were kept at room temperature for 2 hours prior to addition of *M. xanthus*.

Centrifuged cultures of *M. xanthus* strains GJV1, A75 and Serengeti 01 were resuspended to a predicted density of ~10^{10} cells/ml with M9 medium. For each temperature-prey combination (and for control plates), 20 µl of the resuspension were spotted in the middle of the plate and plates were then left open without lids in a laminar-flow hood for 30 min before being incubated at 32 °C and 90% rH. *M. xanthus* swarm diameters were measured after seven days of incubation (two perpendicular diameters per swarm at random orientation). *M. xanthus* swarms of these strains are bright yellow in color. When no evidence of *M. xanthus* growth was observed (*i.e.* no yellow area) after seven days even within the originally-inoculated plate area, a diameter value of zero was recorded. (This occurred only on some plates with *P. fluorescens*).

Test for M. xanthus killing of P. fluorescens reared at 32 °C

For our test of whether *M. xanthus* kills *P. fluorescens* reared at 32 °C on M9cas agar, media and culture-handling protocols were the same as in the swarming assays described above except in the following respects. The killing test was performed on M9cas agar in 50 ml glass flasks rather than in petri dishes. A 10-µl aliquot of resuspended *P. fluorescens* culture was inoculated onto M9cas agar the day before addition of *M. xanthus.* The aliquot was not spread across the plate (as the 600-µl aliquots in the swarming assays were), but was rather allowed to grow into a small circular lawn within the originally inoculated spot area. After incubation for 24 h at 32 °C, a 50 µl aliquot of *M. xanthus* resuspension (~10^{10} cells/ml, as in the swarming assays) was inoculated across the top of and immediately surrounding the circular *P. fluorescens* lawn that had grown up overnight. For the control treatment to which *M. xanthus* was not added, a 50 µl aliquot of M9 liquid was added in the same manner. The flasks were then incubated for four days before *P. fluorescens* was harvested and dilution-plated into LB 0.5% agar, in which *M. xanthus* does not grow. *P. fluorescens* was harvested by adding 1 ml M9 liquid to each flask, scraping the bacteria into

suspension with a loop and mixing the suspension by repeated pipetting to disperse *P. fluorescens* cells. To suspend any cells remaining on the agar after the above procedure, 2.0 ml of additional M9 liquid and 10 glass beads were then added to each flask and flasks were shaken for one hour at 300 rpm, 32 °C prior to dilution plating.

Test for effects of P. fluorescens rearing temperature and inoculum-population size on M. xanthus DK3470 survival

10-ml aliquots of M9cas agar were poured into 50 ml Erlenmeyer flasks one day prior to inoculation and allowed to solidify without flask covers for 30 min in a laminar-flow hood. Flasks were then capped and kept at room temperature overnight. Centrifuged cultures of *P. fluorescens* were resuspended to predicted densities of ~5 x 10^5, ~5 x 10^6 and ~5 x 10^7 cells/ml with M9 liquid. A 200 µl aliquot of resuspended culture was inoculated into each agar flask then spread across the agar surface by gentle rotation. Flasks were then kept open without covers for 60 min in a laminar-flow hood, after which they were capped and flasks of each inoculum population-size treatment were incubated at three temperatures (12, 22 and 32 °C) for 22 hours. After incubation at different temperatures, all flasks were kept at room temperature for two hours prior to either addition of *M. xanthus* or assessment of *P. fluorescens* population size.

For assays of *M. xanthus* strain DK3470 population size*,* 20 µl aliquots of DK3470 culture resuspended to ~10^{10} cells/ml (~2 x 10^8 cells) were spotted in the middle of each flask; flasks were then left open for 30 min in a laminar-flow hood. Flasks were then harvested at one of two time-points: immediately after *M. xanthus* inoculation (and the subsequent 30-min period) and after seven days of incubation at 32 °C. To harvest and disperse DK3470, 10 glass beads and 1 ml M9 liquid were added to each flask and flasks were shaken at 300 rpm, 32 °C for 15 minutes before the resulting suspensions were dilution-plated into CTT 0.5% agar containing gentamicin (10 µg/ml), which prevents growth of *P. fluorescens* but not *M. xanthus*. Colonies were counted after seven days of incubation at 32 °C, 90% rH.

Test for effect of P. fluorescens inoculum population size on post-growth population size

For assays of *P. fluorescens* population size after overnight growth at one of three temperatures, assays that were performed separately from the above-described assays with DK3470, 10 glass beads and 1 ml of M9 liquid were added and the flasks shaken at 300 rpm, 32 °C for 15 minutes to disperse *P. fluorescens* populations. Samples were then dilution-plated into LB 0.5% agar and *P. fluorescens* colonies were counted after two days of incubation at 32 °C, 90% rH.

Test for diffusion of the killing compound(s) produced by P. fluorescens

DK3470 was inoculated onto CTT hard agar from frozen stock four days prior to inoculation in 8 ml CTT liquid in a 50-ml Erlenmayer flask. The resulting liquid culture was grown for ~24 hours at 32 °C, 300 rpm, diluted into fresh medium and grown for another 24 hours before being centrifuged and resuspended to ~5 x 10^9 cells/ml in either supernatant from buffer suspensions of *P. fluorescens* (prepared as described below) or control buffer. The resuspended cultures were incubated for 6 h at 32 °C and then dilution-plated into CTT 0.5% agar. Plates were incubated for 3-5 days before colonies were counted.

To prepare *P. fluorescens* supernatants, *P. fluorescens* was inoculated from frozen stock into 8 ml LB liquid and then incubated overnight at 32 °C, 300 rpm. After dilution to ~5 x 10^7 cells/ml, 200 µl aliquots of the resulting culture were spread across the surfaces of 10-ml aliquots of M9cas agar that had been poured into 50 ml flasks 24 hours before. *P. fluorescens* populations and control flasks containing only M9cas agar were then incubated at 12, 22 and 32°C. After ~24 h, 0.8 ml M9 liquid and ~10 sterile glass beads were added to flasks, which were then shaken at 300 rpm, 32 °C for 15 minutes before 800 µl of each of the resulting culture suspensions were removed from the flask and centrifuged (5000 rpm, 15 minutes). After centrifugation, supernatant was filter-sterilized with 0.2-µm filters. Each sample of filtered supernatant was separated into two equal sub-samples, one of which was heated at 95 °C for 45 min while the other was kept at room temperature.

Test for growth of P. fluorescens on nutrients from M. xanthus killed by P. fluorescens

To test if *P. fluorescens* could grow on nutrients released by *M. xanthus* incubated in *P. fluorescens* supernatant, *P. fluorescens* supernatant was harvested following the same protocol described above, except flasks with *P. fluorescens* lawns were incubated overnight only at 22 °C. *M. xanthus* DK3470 cells from exponential-phase cultures were spun down and resuspended to a density of ~5 x 10^9 cells/ml in the *P. fluorescens* supernatant. These resuspensions with *M. xanthus,* as well as supernatant without *M. xanthus,* were then incubated for 6 hrs, after which i) 10 µl samples from the treatment with DK3470 were plated onto CTT hard agar to test for *M. xanthus* growth after subsequent incubation and ii) 200 µl of M9 liquid culture of *P. fluorescens* at densities of ~10^6 and ~10^8 cells/ml were added to both supernatant treatments (with and without DK3470 resuspended cells). The resulting *P. fluorescens* cultures were then dilution plated onto LB 1.5% agar immediately and after 24, 48 and 96 hours to determine population size.

Test for P. fluorescens supernatant effects on other bacterial species

Erlenmeyer flasks with M9cas agar were prepared as in previous experiments. Centrifuged cultures of *P. fluorescens* were resuspended to a predicted density of 5 x 10^7 cells/ml with M9 liquid, and 200 µl of resuspended samples (or of M9 liquid for the controls) were inoculated into each agar flask and then spread over the agar surface by gentle rotation. Flasks were subsequently kept open

for 60 min in a laminar-flow hood before being closed and incubated at 22 °C for 22 hours. To harvest *P. fluorescens*, 10 glass beads and 0.8 ml of M9 liquid were added to flasks, which were then shaken at 300 rpm, 32°C for 15 min. Suspensions were then centrifuged and supernatant was sterilized with a 0.2 µm filter.

Centrifuged cultures of *A. globiformis, B. bataviensis, C. citreum, E. coli, M. luteus, M. xanthus* strain DK3470*, P. fluorescens,* and *R. vitis* were resuspended to a predicted density of 5 x 10^9 cells/ml with the filtered supernatant, or M9 liquid in the controls. The resulting cell suspensions were incubated at 32 °C, 90% rH for 6 h before they were dilution-plated into CTT (*M. xanthus*) or LB (other species) 0.5% agar. Colonies were counted after 2-4 days of incubation.

Statistical analysis

All analyses were performed using R 4.2.3 (67) as implemented in Rstudio version 2023.03.0+386 (68). We used linear models followed by type III ANOVA (package car (69)) and posthoc contrasts (packages emmeans (70) and multcomp (71)) when appropriate. Figures were created using the package ggplot2 (72). The R script used to analyze this data and the datasets are available on Github at https://github.com/marievasse/Killer-prey and Zenodo (10.5281/zenodo.7823937), respectively.

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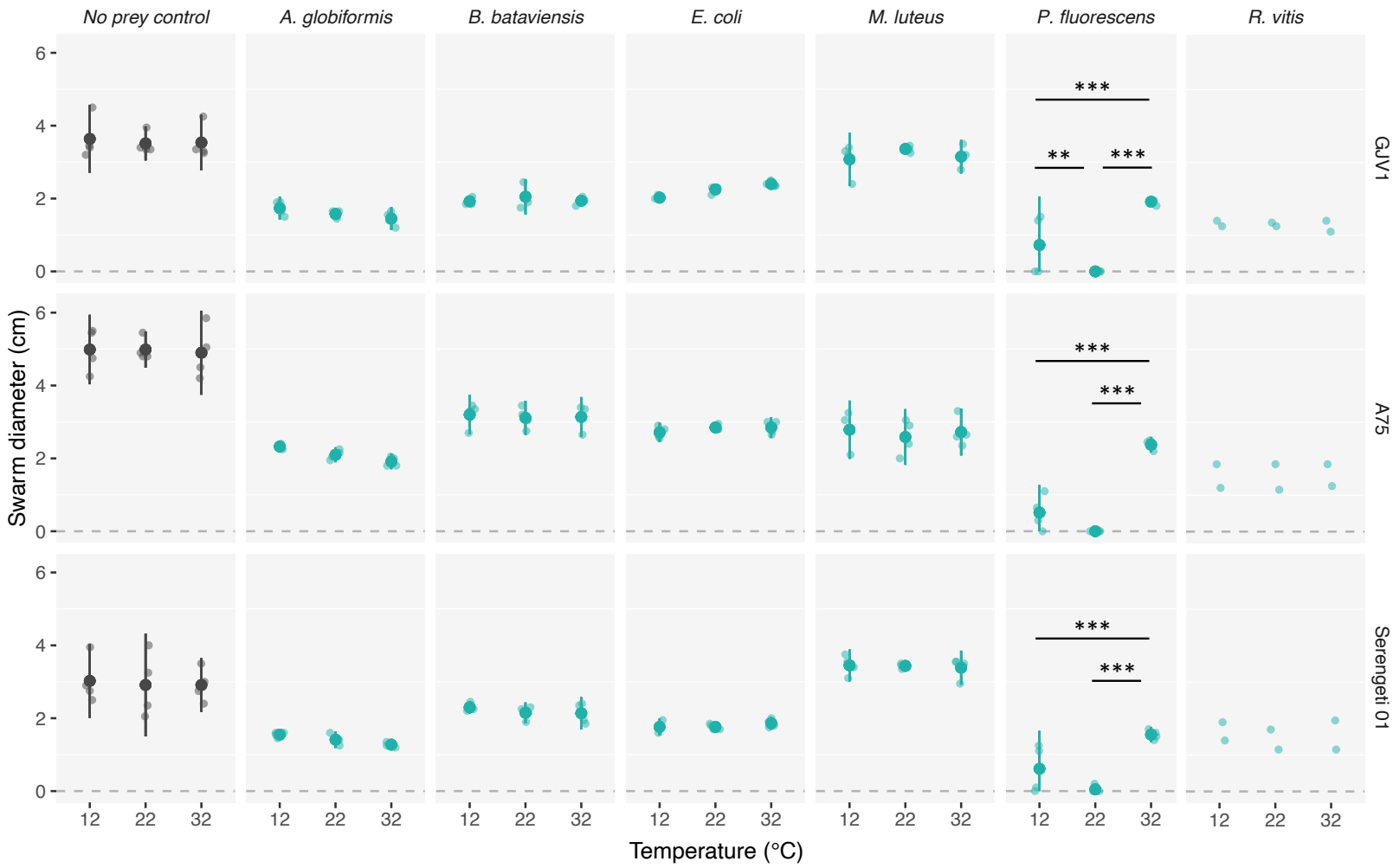
Figures and Tables

Figure 1.


No prey control
A. globiformis
B. bataviensis
E. coli
M. luteus
P. fluorescens
R. vitis
GJV1
A75
Serengeti 01
Swarm diameter (cm)
Temperature (°C)
0
2
4
6
12
22
32
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**
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***
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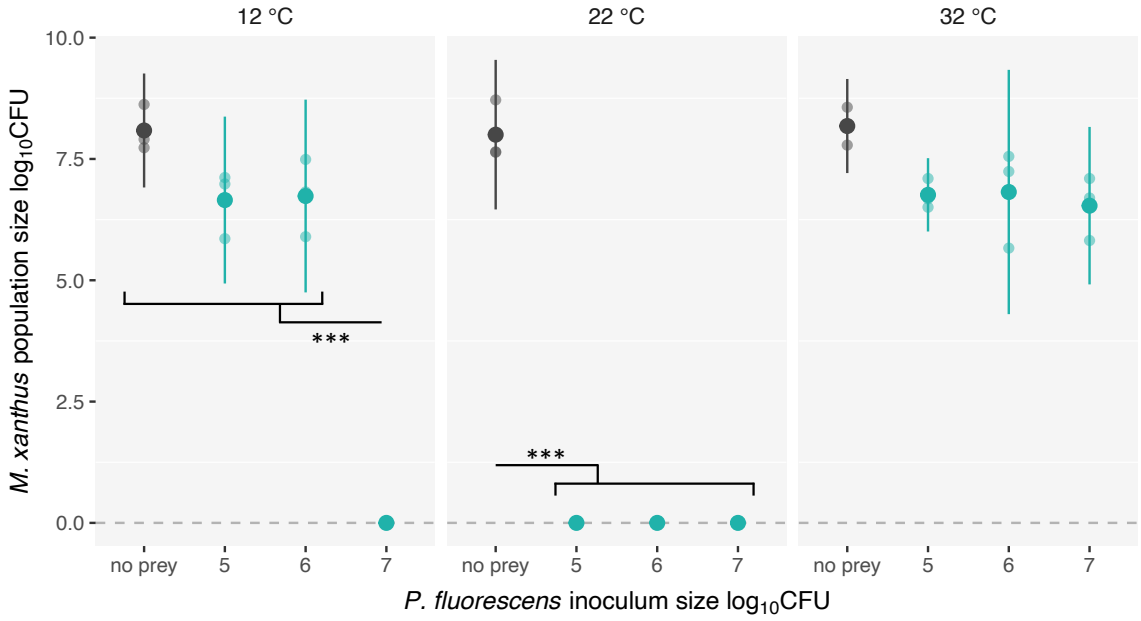
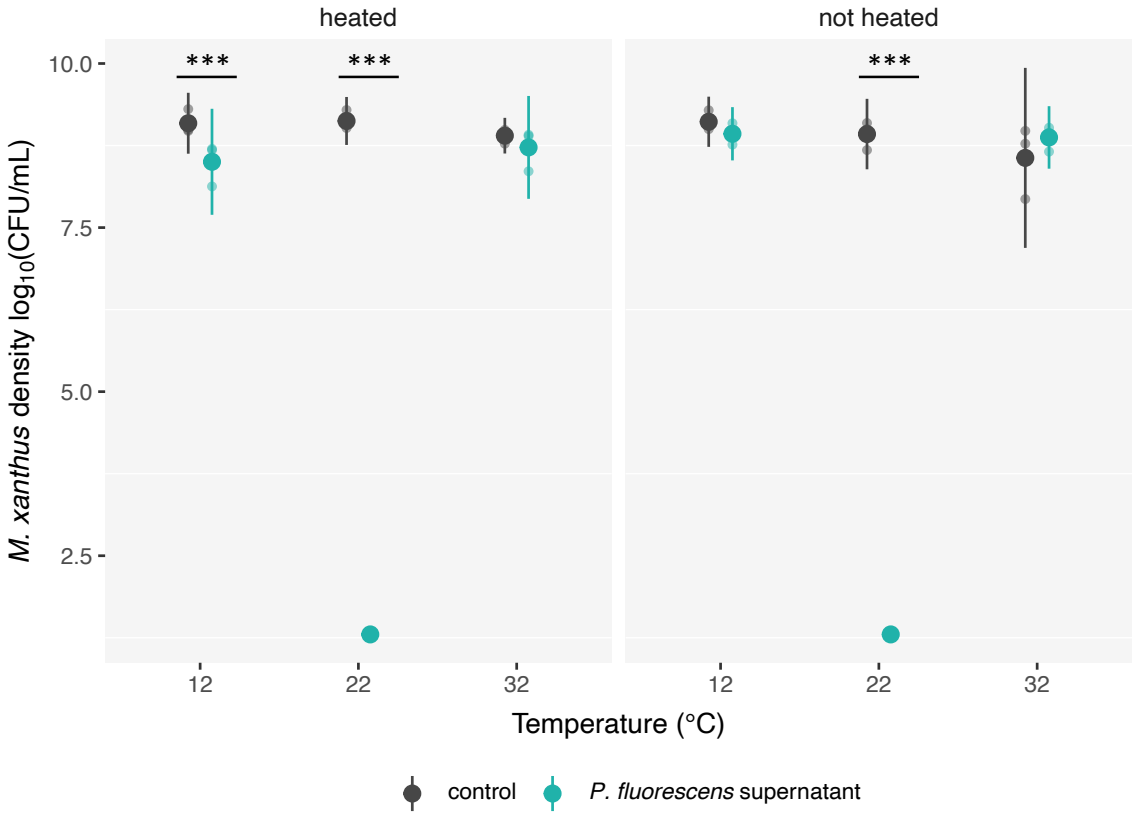
***M. xanthus* swarming through *P. fluorescens* lawns depends on the temperature of *P. fluorescens* growth prior to predator-prey interaction.** Swarm diameters of three *M. xanthus* genotypes (rows) after seven days on M9cas agar bearing either a lawn of one of several prey species (green dots) or no prey (black dots). Prey lawns were incubated at 12, 22 or 32 °C for 22 h and then brought to room temperature for 2 h before *M. xanthus* was added. Small dots are biological replicates (n = 3 except for *R. vitis* for which n = 2), and error bars represent 95% confidence intervals about the means (big dots). Significant differences between average diameters of swarms on prey grown at different temperatures are shown; ** $p < 0.01$, *** $p < 0.001$ (Tukey-adjusted contrasts).

Figure 2.



***P. fluorescens* grown at 22 °C from any inoculum size or at 12 °C from high inoculum size exterminates *M. xanthus*.** *M. xanthus* strain DK3470 population size seven days after inoculation onto *P. fluorescens* lawns that had grown overnight from one of three inoculum population sizes (green dots) at one of three temperatures prior to addition of *M. xanthus*. Black dots show corresponding data for controls lacking *P. fluorescens*. Means of $\log_{10}$-transformed CFU values and 95% confidence intervals are shown. Lighter dots are biological replicates (n = 3). Significant differences between *M. xanthus* population sizes within prey-growth temperature treatments are shown; *** $p < 0.001$ (Tukey-adjusted contrasts).

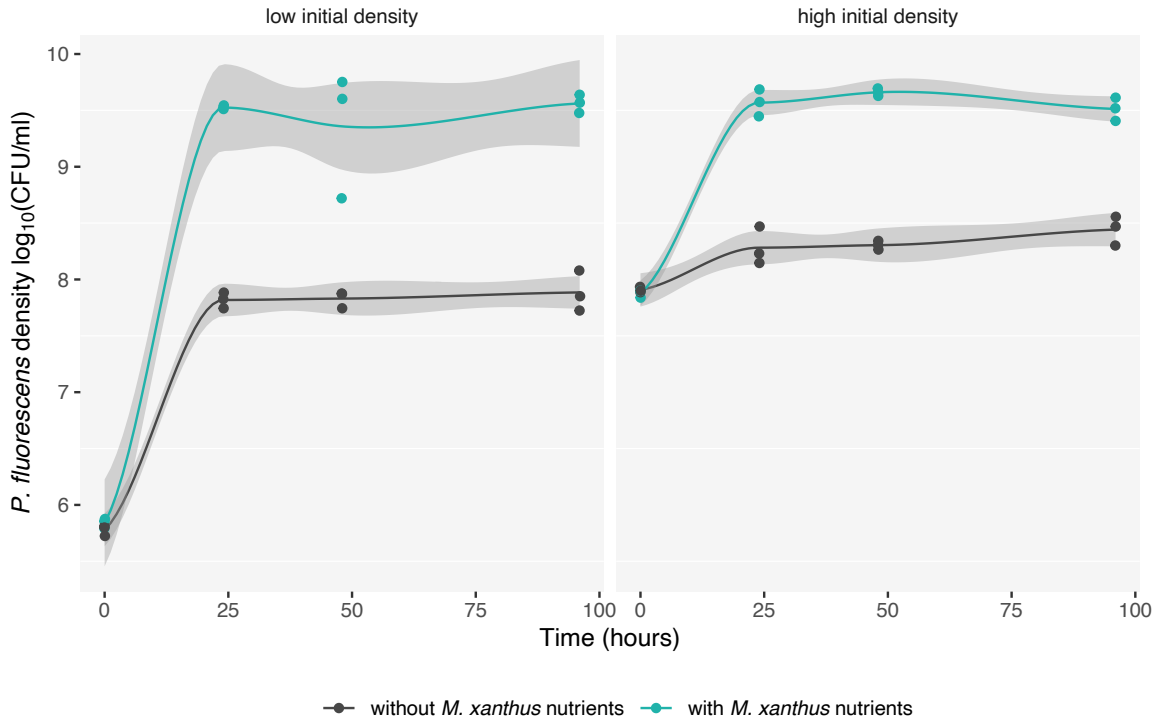
Figure 3.



***P. fluorescens* reared at 22 °C kills *M. xanthus* with a diffusive, non-proteinaceous secretion.**

750 *M. xanthus* density after six hours of incubation in the supernatant of buffer suspensions of *P. fluorescens* cultures grown at 12, 22 or 32 °C for 24 hours (green dots) or in control buffer (black dots) and subsequent exposure to either 95 °C or room temperature for 45' (left and right panels, respectively). Means of $\log_{10}$-transformed CFU/ml values and 95% confidence intervals are shown. Lighter dots are biological replicates (n = 3). Significant differences between predator densities after incubation in supernatant *vs* buffer per prey-growth-temperature treatment are shown; *** p < 0.001 (Tukey-adjusted contrasts).

Figure 4.



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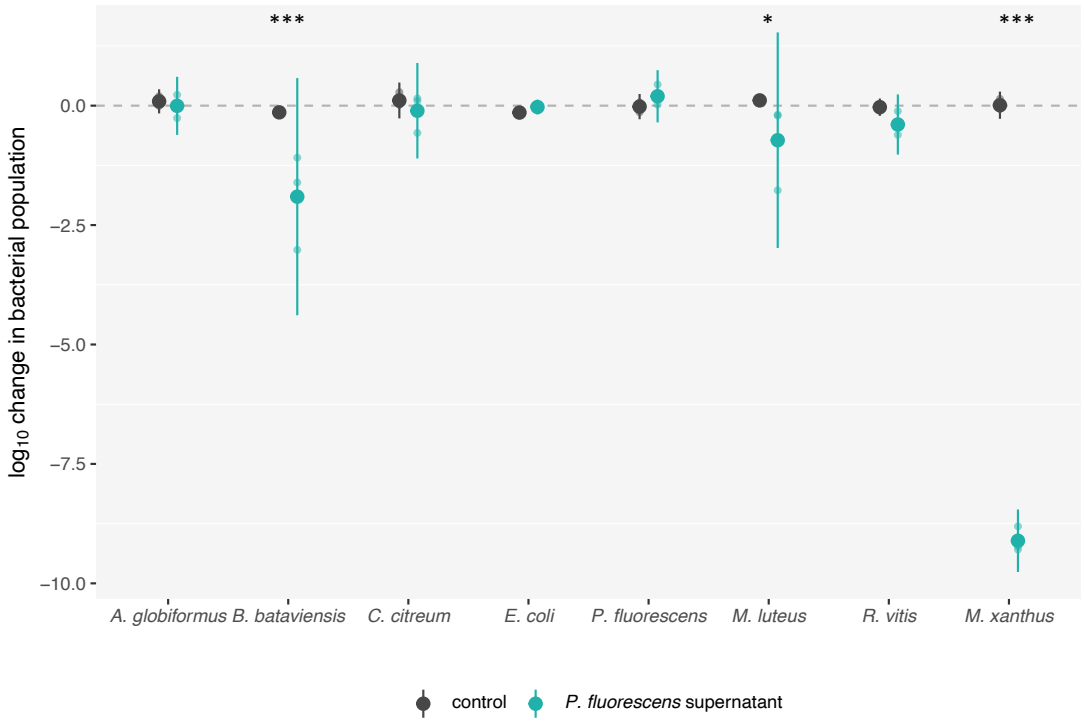
Remains of *M. xanthus* killed by 22 °C-reared *P. fluorescens* fuel large *P. fluorescens* population growth. Estimated densities (log-transformed CFU/ml, *n* = 3) of *P. fluorescens* populations over time inoculated at two initial densities (~10^6 and ~10^8 CFU/ml) into supernatant from 22 °C-reared *P. fluorescens* lawns to which *M. xanthus* cells were either added (and which killed those *M. xanthus* cells, green dots) or not (black dots). Trend lines show local polynomial regression fitting and dark-gray bands represent 95% confidence regions.

Figure 5.



770

Secretions of 22-°C-reared *P. fluorescens* harm a minority of diverse tested species. Change in densities of diverse bacterial species ($\log_{10}$) after six hours of incubation in supernatant from a buffer suspension of *P. fluorescens* grown at 22 °C (green dots) or in a control buffer (black dots). Mean values and 95% confidence intervals are shown. Lighter dots are biological replicates (n = 3). Significant differences between supernatant and control treatments are shown; *** $p < 0.001$ and * $p < 0.05$ (two-sided t tests with Benjamini-Hochberg correction).

Supplementary information for Killer prey: Temperature reverses future bacterial predation

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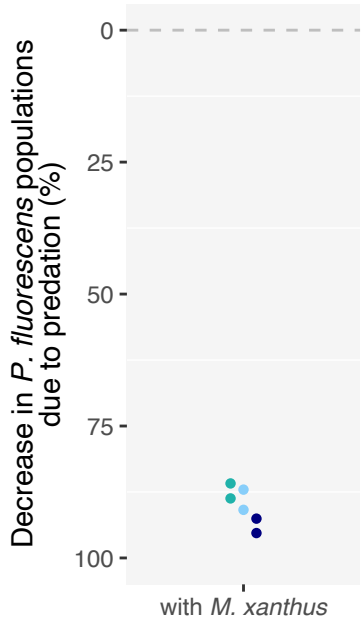
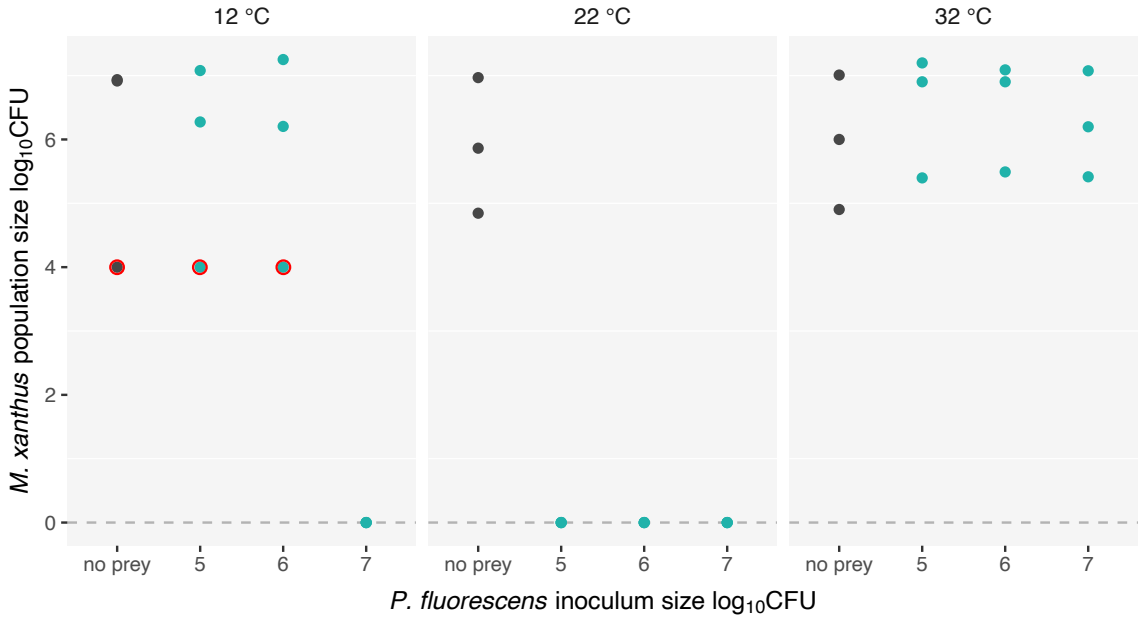
This document contains the Supplementary figures S1-3 and Supplementary tables S1 and S2.

Figure S1.



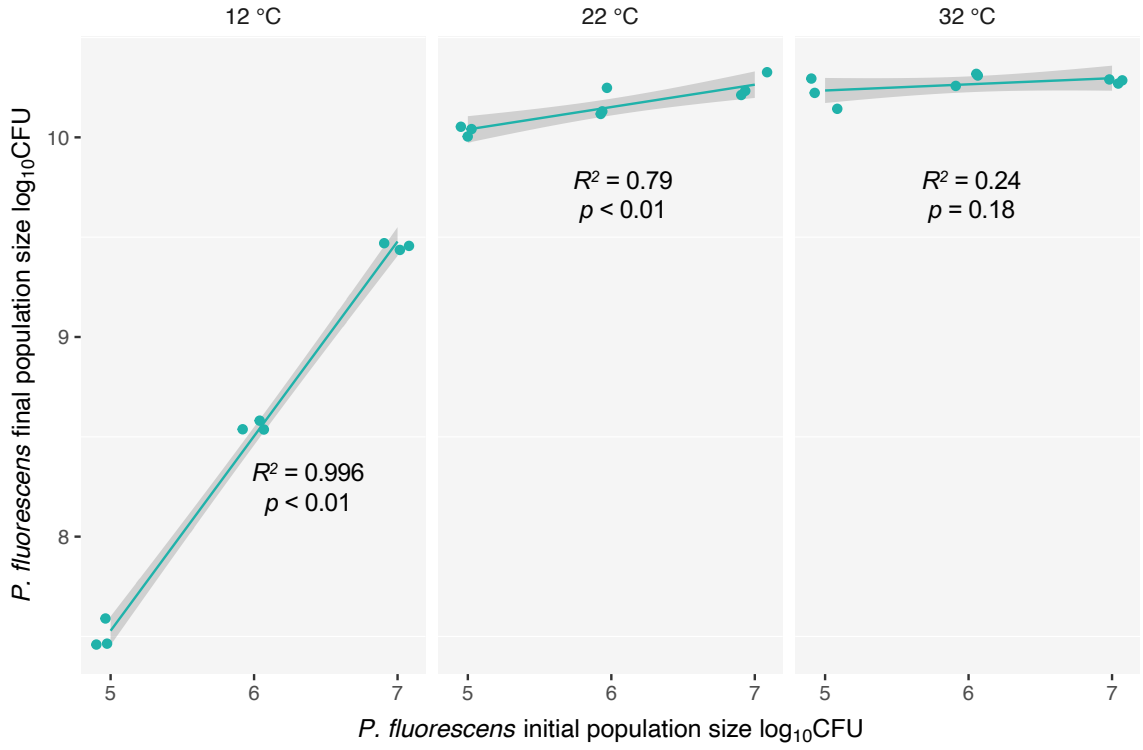
***M. xanthus* kills *P. fluorescens* grown on M9cas agar at 32 °C.** Percentage reduction of *P. fluorescens* population size after four days in the presence of *M. xanthus* relative to in the absence of *M. xanthus*. Colors correspond to three independent replicates each run with two technical replicates.

Figure S2.



***P. fluorescens* grown at 22 °C kills *M. xanthus* after 30 minutes of interaction.** *M. xanthus* population sizes 30 minutes after inoculation onto *P. fluorescens* lawns grown overnight from one of three inoculum sizes and at one of three temperatures (green dots) or onto bacteria-free control plates that had been incubated overnight at one of the same three temperatures prey (black dots). Means of $\log_{10}$-transformed CFU values and 95% confidence intervals are shown. Note that, due to a technical issue with dilution-plating for one replicate of the 12 °C treatment, the highest available plated dilution was too low to accurately count colonies; the corresponding plates had more colonies than could feasibly be counted. We therefore attributed counts of 1000 for these plates, which was clearly a substantial underestimate in each case. These underestimated values are identified in the graph with red circles around corresponding data points. Lighter dots are biological replicates (n = 3).

Figure S3.



Final *P. fluorescens* population size correlates positively with initial population size after growth at 12 and 22 °C but not at 32 °C. Relationship between *P. fluorescens* initial and final population sizes after 22 hours of growth at different temperatures 12, 22 or 32 °C. Log_{10}-transformed CFU values (n = 3), linear fits and 95% confidence intervals about the linear fits are shown.

Table S1. Statistical analysis of *M. xanthus* swarming data on prey lawns. Linear model and Type III ANOVA for swarming data using prey identity, predator identity, temperature treatment and their interactions as explanatory variables. Posthoc contrasts between temperature treatments are computed for each predator-prey combination.

A. Linear model

model <- lm(formula = swarm_diameter ~ prey * predator * temperature)

Multiple R-squared: 0.9325, Adjusted R-squared: 0.908
F-statistic: 38.08 on 62 and 171 DF, p-value: < 2.2e-16

Anova Table (Type III tests)
Response: swarm_diameter

	Sum Sq	Degree of freedom	F value	Pr(>F)
(Intercept)	52.926	1	442.719	< 2.2e-16 ***
prey	22.681	6	31.621	< 2.2e-16 ***
predator	8.065	2	33.733	4.478e-13 ***
temperature	0.035	2	0.146	0.864
prey:predator	10.384	12	7.238	1.234e-10 ***
prey:temperature	7.294	12	5.084	3.151e-07 ***
predator:temperature	0.022	4	0.047	0.996
prey:predator:temperature	1.439	24	0.501	0.975
Residuals	20.442	171		

Signif. codes: 0 = ***, 0.001 = **, 0.01 = *, 0.05 = .

B. Tukey-adjusted contrasts on temperature treatment for each predator-prey combination

pairs(emmeans::emmeans(model, "temperature", by = c("prey", "predator")))

prey = NO, predator = GJV1:

contrast	estimate	SE	df	t.ratio	p.value
12 - 22	0.1250	0.244	171	0.511	0.8660
12 - 32	0.1000	0.244	171	0.409	0.9120
22 - 32	-0.0250	0.244	171	-0.102	0.9943

prey = AG, predator = GJV1:

contrast	estimate	SE	df	t.ratio	p.value
12 - 22	0.1625	0.244	171	0.665	0.7843
12 - 32	0.2875	0.244	171	1.176	0.4691
22 - 32	0.1250	0.244	171	0.511	0.8660

prey = BB, predator = GJV1:

contrast	estimate	SE	df	t.ratio	p.value
12 - 22	-0.1250	0.244	171	-0.511	0.8660
12 - 32	-0.0125	0.244	171	-0.051	0.9986
22 - 32	0.1125	0.244	171	0.460	0.8899

prey = EC, predator = GJV1:

contrast	estimate	SE	df	t.ratio	p.value
12 - 22	-0.2250	0.244	171	-0.920	0.6282
12 - 32	-0.3750	0.244	171	-1.534	0.2777
22 - 32	-0.1500	0.244	171	-0.614	0.8129

Table S2. Statistical analysis of *M. xanthus* growth data. Linear model and Type III ANOVA for *M. xanthus* growth data using *P. fluorescens* inoculum size, temperature treatment, their interaction and time as explanatory variables. Posthoc contrasts between inoculum sizes are computed for each temperature-time combination.

A. Linear model

model <- lm(log10(predator_number +1) ~ prey_inoculum_size * rearing_temperature + time)

Multiple R-squared: 0.9407, Adjusted R-squared: 0.9286
F-statistic: 77.93 on 12 and 59 DF, p-value: < 2.2e-16

Anova Table (Type III tests)
Response: log10(predator_number +1)

	Sum Sq	Degree of freedom	F value	Pr(>F)
(Intercept)	244.066	1	315.709	< 2.2e-16 ***
prey_inoculum_size	192.800	3	83.131	< 2.2e-16 ***
rearing_temperature	0.049	2	0.032	0.969
time	10.421	1	13.480	0.001
prey_inoculum_size:rearing_temperature	195.261	6	42.096	< 2.2e-16 ***
Residuals	45.611	59		

Signif. codes: 0 = ***, 0.001 = **, 0.01 = *, 0.05 = .

B. Tukey-adjusted contrasts on prey inoculum size for each temperature-time combination

pairs(emmeans::emmeans(model, "prey_inoculum_size", by = c("rearing_temperature", "time")))

temperature = 12, time = 30 min:

contrast	estimate	SE	df	t.ratio	p.value
no prey - 5	0.8001	0.508	59	1.576	0.3999
no prey - 6	0.7414	0.508	59	1.461	0.4675
no prey - 7	7.0188	0.508	59	13.826	<.0001
5 - 6	-0.0587	0.508	59	-0.116	0.9994
5 - 7	6.2187	0.508	59	12.250	<.0001
6 - 7	6.2773	0.508	59	12.366	<.0001

temperature = 22, time = 30 min:

contrast	estimate	SE	df	t.ratio	p.value
no prey - 5	6.9464	0.508	59	13.684	<.0001
no prey - 6	6.9464	0.508	59	13.684	<.0001
no prey - 7	6.9464	0.508	59	13.684	<.0001
5 - 6	0.0000	0.508	59	0.000	1.0000
5 - 7	0.0000	0.508	59	0.000	1.0000
6 - 7	0.0000	0.508	59	0.000	1.0000

temperature = 32, time = 30 min:

contrast	estimate	SE	df	t.ratio	p.value
no prey - 5	0.4443	0.508	59	0.875	0.8176
no prey - 6	0.4176	0.508	59	0.823	0.8435
no prey - 7	0.6910	0.508	59	1.361	0.5284
5 - 6	-0.0267	0.508	59	-0.053	0.9999
5 - 7	0.2467	0.508	59	0.486	0.9619
6 - 7	0.2734	0.508	59	0.539	0.9492