Prey attraction in a generalist microbial predator, *Dictyostelium discoideum*

P. M. Shreenidhi, Rachel I. McCabe, David C. Queller, Joan E. Strassmann

Department of Biology, Washington University in St. Louis, St. Louis, Missouri, USA

Corresponding author: P. M. Shreenidhi

Corresponding author email: shreenidhi@wustl.edu

Keywords: Innate preference, Chemotaxis, Learning, Bacterial prey

Author contributions: P.M.S., D.C.Q., and J.E.S. designed research. P.M.S., and R.I.M. performed research. P.M.S., D.C.Q., and J.E.S. analyzed the data. P.M.S., D.C.Q., and J.E.S. wrote the paper with inputs from R.I.M.

Competing Interest Statement: The authors declare no competing interests.

This PDF file includes:

Main Text

Figures 1 to 3

Tables 1

Supplementary figures 1 to 4

Abstract:

Generalist predators often live in environments that vary in the type and abundance of prey species. The prey species in turn vary in their susceptibility and suitability to predation. How do generalists navigate this variation in prey abundance and profitability and seek out their preferred prey? We investigated this in the soil protist *Dictyostelium discoideum*, a generalist predator of many species of bacteria. Despite their generalist diet, amoebas experience considerable variation in growth rate on their prey bacteria. We tested if amoebas innately prefer the more profitable prey bacteria. We found that the levels of chemoattraction of naïve amoebas towards prey bacteria increased with increasing growth rate of the amoebas on those bacteria. This suggests that *D. discoideum* amoebas have an innate prey preference that is adaptive. Next, we tested how experience with prey bacteria affects chemoattraction in amoebas. Given the number of bacterial species in soil, learning from experience should be advantageous. However, we found no evidence for learning; instead amoeba preference in learning experiments was partially explained by innate attractiveness of the prey bacteria. Our results suggest that generalist amoebas are innately attracted to the more profitable prey bacteria and this innate attraction cannot be overridden by recent experience.

Introduction:

Predation, where one individual kills and consumes another, is a fundamental interaction between organisms which can profoundly shape organismal evolution. One key component of a predator's fitness is the ability to sense its prey. Specialist predators are often superior at detecting their prey as compared to generalists (Bernays 2000). Since specialist predators feed on few prey species, they need to track fewer features of their environment to quickly and accurately detect their preferred prey. But generalist predators feed on a diversity of prey

species. This very abundance of choice may hinder their ability to detect relevant information about their prey.

Generalist predators, or more broadly, generalist consumers, can overcome this problem either by innate or learned mechanisms. Generalists might possess innate preferences for certain environmental features, such as shape or color, that can drive foraging decisions. For example, though honey bees, *Apis mellifera*, are generalist pollinators of many flower species, they exhibit an innate preference for some colors (Giurfa et al. 1995). Similarly, the generalist hoverfly, *Eristalinus aeneus*, is attracted to artificial floral objects that are bright, radially symmetrical, and possess plant odors (Mishra et al. 2024). Plasticity in behavior and the ability to learn from experience are also critical components of foraging behavior in generalists (Bernays 2000; Mery and Burns 2010). Learning can increase the efficiency of decision making by generalists and allow them to track the most profitable or abundant resources. For example, honey bees, despite their preference for some colors, can quickly be trained to prefer alternative colors if associated with rewards (Giurfa et al. 1995). Many avian predators learn to avoid chemically defended insects based on their warning coloration (Guilford 1990).

Prey preference has been extensively studied in macroscopic predators such as birds or insects that rely on visual and olfactory cues to track their prey (Guilford 1990; Dukas 2008). These preference behaviors are encoded in the nervous system. But the ability to track prey is crucial even to microscopic organisms that lack a nervous system. Soil protists are often generalist predators that feed on diverse prey bacteria (Johnke et al. 2014; Amacker et al. 2022). Protist predation is a major cause of bacterial mortality in the environment (Jousset 2012). Predation by protists can shape bacterial communities, facilitate nutrient cycling and drive the evolution of bacterial defenses (Sherr and Sherr 2002; Jousset 2012; Smith et al.

2023). However, protist predation must require fundamentally different methods for prey detection compared to macroscopic predators.

Prey detection can be challenging for soil protists because of the complexity of soil environments. Soil is a heterogenous environment that varies spatially and temporally in the abundance and diversity of prey bacteria. Even a gram of soil is extremely diverse and may contain anywhere between two thousand and eight million bacterial species (Gans et al. 2005; Schloss and Handelsman 2006). From a protist's perspective, soil is composed of minute particles of clay, sand, and decayed plant material separated by water and air-filled pores (Vos et al. 2013).The particles and pores lead to a patchy distribution of resources resulting in fragmented bacterial communities. Many bacteria reside in these micro-pores and are protected from predation. Protists may need to navigate this maze-like soil habitat to seek out their preferred prey bacteria.

Dictyostelium discoideum, also known as the social amoeba, is a protist predator well-known for its fascinating multicellular cycle (Strassmann and Queller 2011). *D. discoideum* in its simplest form persists as unicellular amoebas in temperate forest soils where they are generalist predators of soil bacteria. The prey bacteria are diverse and range across at least four bacterial phyla: Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Brock et al. 2018). Despite being generalist predators, these amoebas experience variation in growth rate on these prey bacteria (Shreenidhi et al. 2024). Under laboratory conditions, they can double in three hours on some of the most edible species of bacteria and take as long as ten hours to double on the less edible species. Some species of bacteria are plainly inedible, while some others are pathogenic and can infect the multicellular fruiting bodies (Brock et al. 2018; Steele et al. 2023). Thus, *D. discoideum* interacts with bacteria in complex ways ranging from being a formidable predator to a susceptible host.

The edibility of a bacterium is a joint phenotype (Queller 2014) of the amoeba's offense and bacterial defense. Bacteria possess an arsenal of defenses to fend off protist predation (Jousset 2012; Smith et al. 2023). Bacteria can secrete diffusible toxins or repellents to foil the search by the predator. They can modify surface properties and seek refuge to evade encounter and recognition. Bacteria can form secondary structures such as biofilms, filaments, and microcolonies to deter attack and ingestion. Finally, bacteria can produce toxins and use secretion systems to prevent digestion. Thus, given the variation in bacterial profitability and defensive responses, it would be advantageous if the amoebas could sense the suitability of a bacterium for predation from a distance or modulate their preference based on their prior experience with a prey bacterium.

D. discoideum amoebas use chemotaxis to locate their prey bacteria (King and Insall 2009). Amoebas are attracted to bacterially secreted cyclic adenosine monophosphate (cAMP) (Meena and Kimmel 2017; Rashidi and Ostrowski 2019) and folic acid (Pan et al. 1972). These two chemoattractants are relayed through two different G-protein coupled receptors, cAR1 and fAR1 respectively. *D. discoideum* amoebas are also known to be more attracted on average to Gram-negative bacteria compared to Gram-positive bacteria (Rashidi and Ostrowski 2019). But the relationship between bacterial edibility and innate attraction of amoebas remains to be tested since there is wide-variation in edibility even within gram-negative bacteria (Brock et al. 2018; Steele et al. 2023; Shreenidhi et al. 2024).

There are multiple reasons why experience with prey bacteria may play an important role in modulating prey preference in amoebas. It is unlikely that amoebas can evolve a perfect innate response to all prey bacteria given the number of bacterial species in soil. Amoebas may learn from experience to be more attracted to profitable bacteria and avoid unprofitable or harmful bacteria. Prey experience might also modulate prey preference because of the cost

of prey-switching in amoebas (Shreenidhi et al. 2024). Amoebas undergo prey-switching costs where they experience a reduction in growth rate when switched from one species of prey bacteria to another compared to controls that did not experience a prey switch. These costs are likely physiological and related to changing transcriptional response to match the new prey bacterium. Since switching from a familiar bacterium to a novel bacterium is associated with costs, it is possible that experience with a prey bacterium primes amoebas to be more attracted towards that bacterium compared to others.

In this study, we measured the innate preference of *D. discoideum* amoebas towards phylogenetically diverse species of prey bacteria that were previously isolated from close association with *D. discoideum* from soil environments. We then tested if amoebas are more attracted to the more edible prey bacteria. Next, we looked at how experience or familiarity with a prey bacterium affects prey preference in these amoebas. We tested if amoebas displayed any evidence of learning such that they are differentially attracted to a bacterium if they have prior experience of it being profitable prey.

Methods:

Growth rate data:

To test for correlation between innate attraction and growth rate of amoebas on different bacteria, we used previously published growth rate data of *D. discoideum* for 20 of the 24 bacterial species (Shreenidhi et al. 2024). We used similar assay protocols to estimate growth rate of *D. discoideum* on our other four bacterial species. We estimated the Malthusian parameter **r** over 20 h on plates.

Innate prey preference experiments:

We performed the innate preference experiments on AX4 amoebas that had been grown axenically on HL5 medium including glucose (Formedium) with antibiotics (300 µg/ml Streptomycin, 100 µg/ml Carbenicillin), free of any prey bacteria. This was to remove any effects of learned preference for recently experienced bacteria. Amoebas were inoculated at a starting concentration between $5 \times 10^5 – 2 \times 10^6$ cells/ml in 25 ml of HL5. The flasks were placed in a shaking incubator at 21ºC, 100 rpm. The amoebas were transferred into fresh HL5 media every 3-4 days to maintain them at a concentration below 2×10^7 cells/ml. We collected amoebas for the experiment by centrifuging the cultures at 300*g*, 10ºC for 3 minutes. We discarded the spent media and washed the amoeba pellet once more in KK2 buffer (2.25 g KH_2PO_4 (Sigma-Aldrich) and 0.67 g K_2HPO_4 (Fisher Scientific) per liter). The amoeba pellet was suspended in 2 ml KK2 buffer. We estimated amoeba concentration using a hemocytometer and diluted them to a final concentration of 10^7 cells/ml for the experiment. The bacteria for the experiments were grown on LB (Fisher Scientific) or SM/5 plates (2 g glucose (Fisher Scientific), 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgSO_4$ (Fisher Scientific), 1.9 g KH_2PO_4 (Sigma-Aldrich), 1 g K_2HPO_4 (Fisher Scientific), 15g agar (Sigma-Aldrich)), and resuspended to a concentration of OD_{600} 5 in KK2 buffer.

We used assay protocols similar to (Rashidi and Ostrowski 2019) for the chemotaxis experiments. The experiments were performed in 6-well plates containing 2 ml of 2% noble agar in KK2 buffer per well. In the center of each well, we pipetted a 2µl droplet of the amoeba suspension (10^7 cells/ml). A 2 µl spot of the bacterial suspension was placed at 0.65 cm on one side of the amoeba spot and a 2 µl droplet of KK2 buffer at the same distance on the other side (Figure 1A). The spots were placed using a template. We measured the chemotaxis of AX4 amoebas towards 23 species of soil bacteria that had been collected in close association with *D. discoideum* and *Klebsiella pneumoniae*, with three technical replicates per bacterium (Table 1). The experiments were performed over four different days.

We repeated chemotaxis experiments towards *Klebsiella pneumoniae* on all days to account for any day-specific effects. We imaged (phase contrast, 10x magnification) the plates at 6 h from set-up using the Leica Thunder Live Cell Microscope with an automated stage.

Learned prey preference experiment:

We studied learned prey preference in a wild strain of *D. discoideum,* QS9 isolated from Mountain Lake Biological Station, Virginia in 2000 and then stored in a -80 °C freezer (Fortunato et al. 2003). To test how experience with a prey bacterium affects prey preference, we first "familiarized" the amoebas to that bacterium. We chose eight bacteria on which *D. discoideum* has a high growth rate so that the familiar bacterium provides a positive stimulus. We spread 200 µl of 1.5 OD_{600} bacterial suspension on SM/5 plates and pipetted 50 µl of spore solution containing 200,000 spores. The amoebas were collected from the resulting vegetative front after 3 - 4 days with a sterile loop. For some bacterial species where the front was hard to see, we collected amoebas from the entire plate after ~ 36 - 40 hours. We suspended the amoebas in KK2 buffer and centrifuged them at 300*g*, 10 ºC for 3 minutes. We washed the amoeba pellet three more times to remove any bacteria. We used the same chemotaxis set-up as described in the "Innate prey preference experiments", with a central spot of amoebas flanked by two spots of potential attractants. But here the amoebas were given a choice between the bacterium they were grown on ("familiar bacterium") and a new bacterium ("novel bacterium") (Figure 1B). We did this experiment with eight bacterial species in a full factorial design. Amoebas grown on each familiar bacterium were tested against seven novel bacteria (Table 1, bacterial species highlighted in green). All experiments for amoebas conditioned to a given familiar bacterium were done on the same day, with three technical replicates for every choice between familiar and novel bacterium. We imaged the plates at both 4 h and 6 h from set-up.

Image Analysis:

We used ImageJ version 1.53 (Schneider et al. 2012) to estimate number of amoebas that moved out of the amoeba spot towards each of the two potential attractants. We drew a tangent to the amoeba spot at a point that was closest to each potential attractant to define a boundary and counted only those amoebas that moved past this boundary (Figure 1A) (Rashidi and Ostrowski 2019). We did the following image processing steps: *Convert to 8-bit > Set Threshold > Make Binary > Fill holes > Apply Watershed*. We used the *Analyze particles* feature on the relevant parts of the image to count the number of cells.

Data analysis & Statistics:

Innate prey preference experiments:

To estimate net amoeba movement towards a given bacterium, we subtracted amoeba movement towards KK2 buffer from amoeba movement towards the bacterial spot. To account for day-specific effects (Figure S1), we scaled all amoeba movement estimated on a given day by dividing it by net amoeba movement towards *Klebsiella pneumoniae* (averaged across 3 replicates) on that day. We refer to these values corrected for both buffer and day effects as normalized amoeba movement.

To investigate the relationship between innate preference of amoebas and edibility of the bacterium, we needed to control for the phylogenetic non-independence of data points. We used a phylogeny including all 23 species of soil bacteria used in our experiments as a random factor in our models to control for this. These bacteria are a subset of a larger collection of bacterial strains isolated from close association with *D. discoideum* (Brock et al. 2018). We reconstructed the tree in Brock et al. 2018 by using the previously published 16s sequences of the bacteria (deposited by Brock et al. on GenBank) and 26 species of anchor bacteria (full length 16s sequences downloaded from SILVA rRNA database). A multiple

sequence alignment of all sequences was generated using MUSCLE on Geneious Prime. IQ-TREE version 2.3.6 was used to construct a maximum-likelihood tree with model optimization and 100 bootstraps to assess branch support (Minh et al. 2020). We pruned the tree to the 23 species of bacteria used in chemotaxis experiments using iTOL (Letunic and Bork 2021) and used this tree in newick format for all downstream analysis. We forced the phylogenetic tree to be ultrametric using *chronos* function (lambda = 1) in *ape* package in R (Paradis and Schliep 2019) and used *inverseA* function in *MCMCglmm* package (Hadfield 2010) to convert the phylogeny into a covariance matrix.

We then used the R package *MCMCglmm* (Generalized linear-mixed model models using Markov Chain Monte Carlo approach) to examine the relationship between normalized amoeba movement (response variable, henceforth referred to as innate preference) and mean growth rate of amoebas (fixed effect) and controlled for phylogenetic relationships among the bacteria by modelling the bacterial phylogeny as a random effect. We used default prior for the fixed effect and weakly informative priors for residual variance and random effects (V=1, nu = 0.002) to not overly influence posterior distributions. We report the pMCMC values and the R^2 for the fixed effect calculated as described using (Nakagawa and Schielzeth 2013).

To test for chemokinesis (stimulation of undirected movement), we looked for correlations between average movement towards buffer (response variable) and average movement towards bacterium (fixed effect), scaled as before by average movement towards *Klebsiella pneumoniae* on a given day. We used a MCMCglmm to model this with Bacterial phylogeny as the random factor.

Learned preference experiments:

We counted the number of amoebas moving towards the familiar bacterium or novel bacterium in each replicate at 4 h and 6 h from set-up. We used the average from these two time points for each replicate for downstream analysis. Replicates with zero total movement were removed from the analysis. Next, we converted these numbers into proportional movement for each replicate: proportion of all moving amoebas that moved towards familiar bacterium i, when paired with novel bacterium j. We averaged across the replicates to calculate $f_{i,j}$ for each pairing.

We estimated average proportional movement towards bacterium i when familiar (F_i) as:

$$F_i = \frac{\sum_{j \neq i} f_{i,j}}{7}$$

We estimated average proportional movement towards bacterium i when novel (N_i) as:

$$N_i = \frac{\sum_{j \neq i} (1 - f_{j,i})}{7}$$

For a given bacterium we have two proportional amoeba movement measures, one when the bacterium is familiar and another when the bacterium is novel.

We tested if proportional movement towards familiar bacterium (collection of $f_{i,j}$'s) is significantly different from 0.5 using a linear model with familiar bacterium as fixed effect and arcsine transformed proportional movement data as response variable. We used *emmeans* package to calculate the estimated marginal means and confidence intervals (Lenth et al. 2022). To test if learned preference of amoebas is correlated with innate preference, we built a MCMCglmm model with innate preference towards prey bacterium (data from the innate preference assay) as fixed effect and average proportional movement towards bacterium when familiar (F_i) as the response variable. To test if experience with a prey bacterium affects chemoattraction of the amoebas to the bacterium, we calculated F_i (average movement

when familiar) - N_i (average movement when novel) for all 8 species of bacteria and tested if this was significantly different from zero using MCMCglmm. Since these data are also collected across different species of bacteria, we added in the phylogeny of the 8 bacterial species assayed as a random effect. We followed the same steps as described previously to construct the phylogeny, except we pruned the phylogenetic tree down to the 8 species of bacteria used in the learned preference experiments. We used the default priors for the fixed effect and weakly informative prior for residual variance and random effects (V=1, nu = 0.002) to not overly influence posterior distributions. We report the pMCMC values and the R^2 for the fixed effect calculated as described using Nakagawa & Schielzeth 2013.

Results:

Can amoebas preferentially detect and direct movement towards better prey?

To estimate innate prey preference in *D. discoideum* amoebas, we measured the movement of naïve amoebas towards 23 species of soil bacteria or towards KK2 buffer (Figure 1A, Table 1). Amoeba movement was generally directed towards the bacteria and rarely towards buffer (Figure S2). As our measure of innate preference, we subtracted movement towards KK2 from movement towards bacteria and scaled all amoeba movement by movement towards *Klebsiella penumoniae* on the same day to get normalized net amoeba movement towards different species of bacteria (Figure S3). To test if innate response was adaptive, we tested the relationship between normalized movement and growth rate of amoebas on these bacteria correcting for the bacterial phylogeny. We found that normalized movement increases with increasing growth rate (Figure 2; MCMCglmm, Fixed effect posterior mean = 4.58, 95% CI (1.00, 8.11), pMCMC = 0.011, fixed effect R^2 = 0.21). Thus, amoebas possess an adaptive innate response where they tend to be innately attracted to more profitable bacteria.

While chemotaxis is the directed movement of cells in response to a chemical gradient, chemokinesis refers to random undirected movement in response to chemical cues. If some bacteria release chemokines that increase amoeba movement overall, then we might observe an increase in amoeba movement in all directions (though presumably weaker at the far end of the amoeba spot). To test if chemokinesis can explain the observed movement patterns, we tested if movement towards KK2 buffer was correlated with movement towards bacteria. We found no significant relationship between these two measures suggesting that these patterns are not driven by chemokinesis (Figure S4: MCMCglmm, Fixed effect posterior mean = 0.04, 95% CI (-0.28, 0.10), pMCMC = 0.24).

How does experience with prey bacteria affect preference in amoebas?

Because the innate effect explains 21% of the variance, there is plenty of scope for improvement via learned effects. To test if prior experience with prey bacteria affects preference in amoebas, amoebas were familiarized to different bacteria and were then given a choice between the bacterium they were grown on (familiar bacterium) or new bacterium (novel bacterium) (Figure 1B). We chose eight bacteria on which *D. discoideum* has a high growth rate so that the familiar bacterium provides a positive stimulus, and tested whether amoebas would learn and be more attracted to this good prey. We found wide variation in proportional attraction of amoebas towards the familiar bacterium (Familiar bacterium: $F_{8,48}$ = 2.81, p-value = 0.012), with no obvious overall trend of preference for the familiar bacterium (Figure 3A). For example, when amoebas were familiarized to *Pseudomonas kuykendalli* (*Pk*), they seemingly prefer the familiar bacterium (*Pk*) over the novel bacteria (Estimated marginal means = 0.13, 95% CI (-0.006, 0.27), p = 0.06). However, when amoebas were familiarized to *Variovorax boronicumulans* (*Vb*), they preferred all the novel bacteria over the familiar bacterium (*Vb*) (Estimated marginal means = -0.18, 95% CI (-0.32, -0.04), p = 0.01).

However, since innate attraction to bacteria influences learned preference (Figure 3B; MCMCglmm, Fixed effect posterior mean = 0.16, 95% CI (-0.02, 0.36), pMCMC = 0.07, R^2 = 0.28), we cannot determine if prey experience modulates preference by merely comparing proportional movement of amoebas towards bacterium when familiar to the baseline value of 0.5. Because our experiments employ a fully factorial design, each prey bacterium appears in both familiar and novel treatments. Therefore, we accounted for innate preference for the bacterium by comparing the average movement towards bacterium when it is familiar to average movement towards the bacterium when it is novel ($F_i - N_i$). The difference between the average familiar and novel proportions was not different from zero and the trend is in the direction opposite to learning (Figure 3C; MCMCglmm, Fixed effect posterior mean = -0.08, 95% CI (-0.19, 0.04), pMCMC = 0.151).

Discussion:

All predators must find prey, but generalists that have many suitable prey may have the most difficulty evolving rules to identify them and distinguish them from unsuitable prey. This may be especially true for microbial predators that encounter many potential bacterial prey species and lack nervous systems to process the information. In this paper, we tested for both innate and learned prey preferences of *D. discoideum* amoebas towards their prey bacteria, as measured by chemoattraction. We found that naïve amoebas are more attracted, on average, to prey bacteria that are more profitable. Thus, innate prey preferences of *D. discoideum* amoebas are generally adaptive. This result hints at the long evolutionary history of amoeba-bacteria interactions and how these innate responses have been selected to enhance fitness over evolutionary time. But as with any correlation we cannot establish the direction of causation between innate preference and prey profitability. It seems likely that variation in amoeba performance on different bacteria shaped prey preferences. But it is also possible that amoebas improved their performance on bacteria that were already attractive.

Both traits likely influenced each other's evolution and there are bound to be other evolutionary constraints. For example, *D. discoideum* amoebas use chemotaxis not only to locate their bacterial prey, but also to locate other conspecific amoebas during starvation to initiate the multicellular cycle. Thus, innate chemoattraction towards bacterial prey cannot be changed to the extent that it hinders the ability of the amoebas to find other conspecifics. Indeed, cAMP is a common chemoattractant signal used by *D. discoideum* to detect both bacterial prey and conspecifics using the chemoreceptor cAR1 (Klein et al. 1988; Meena and Kimmel 2017; Rashidi and Ostrowski 2019).

A greater constraint is prey secretions, prey profitability, and prey defense. Because prey bacteria are constantly evolving, it seems impossible to evolve innate preferences that remain adaptive on all encountered bacterial species. Our data shows that only 21 % of variance in amoeba attraction is explained prey profitability suggesting that there is more scope for perfecting this response. Some behavioral plasticity in prey preference and learning from experience is likely to be adaptive.

The question of whether single-celled organisms can learn from experience and change their behavior through associative conditioning used to be a hotly debated topic in the 20th century (Gershman et al. 2021; Reid 2023). But there has been growing evidence and acceptance that single-celled organisms are capable of associative learning. Motility patterns consistent with associative learning were observed in *Amoeba proteus*, where the amoebas learned to associate the electric field, an unrelated conditioning stimulus, with a bacterially secreted chemotactic peptide, the unconditioned stimulus. Similar associative learning was also observed in *Amoeba borokensis* and *Metamoeba leningradensis* (Carrasco-Pujante et al. 2021).

However, we found that prior experience with a prey species did not generally seem to influence subsequent attraction of *D. discoideum* amoebas to that bacterium. Because these were all profitable prey species, we expected amoebas might learn to be more attracted to them, but they did not. The idea that amoebas can change their prey preference based on experience rests on the assumption that the prey bacteria secrete different chemoattractants, or combinations of chemoattractants, and that amoebas can distinguish between them, probably by using different chemoreceptors or combinations of chemoreceptors. Experience with a prey bacterium could then lead to upregulation of those specific chemoreceptors, or to upweighting the information from those chemoreceptors, thus reinforcing preference. Not much else is known about prey chemoattractants of *D. discoideum* apart from folate and cAMP (Artemenko et al. 2014). The response to these chemoattractants is mediated by two highly-ligand specific G-protein coupled receptors (GPCRs), the cAR1 receptor for cAMP and the fAR1(GrlL) receptor for folate (Pan et al. 2016)(Meena and Kimmel 2017). But amoebas possess 61 putative receptors in the GPCR family (Heidel et al. 2011). Many of these potential GPCRs are of unknown function and we had expected that some of them could be undiscovered chemoreceptors that may play a role in modulating experience based on experience. All that would be needed is a GPCR with a different ligand binding domain outside the membrane but the same signaling machinery inside the membrane to stimulate actin remodeling to support chemotaxis.

Though we observed no consistent change in amoeba response based on experience with prey, variation in amoeba movement in learning experiments was partly explained by their innate preference for a specific bacterium. It is possible that the bacteria assayed in our experiments do not secrete sufficiently different chemoattractants, and that amoebas only use folate and cAMP to detect their prey bacteria. We know that amoeba attraction towards these

two chemoattractants is dose-dependent where higher doses of the attractant lead to more amoeba movement (Meena and Kimmel 2017; Rashidi and Ostrowski 2019). Thus, it is possible that the prey bacteria we tested vary in the levels of cAMP or folate production and that amoebas simply prefer prey bacteria that produce more cAMP or folate, irrespective of prey experience.

The levels of environmental heterogeneity experienced by an organism plays an important role in the evolution of innate and learned responses (Mery and Burns 2010). Innate behavioral responses evolve when the environment is relatively stable over several generations, while plastic/learned responses evolve when the environment changes rapidly over the organism's lifetime (Stephens 1991). The presence of an innate adaptive prey preferences and the lack of evidence for learning in our experiments suggests that perhaps amoebas do not experience very much environmental heterogeneity in prey species. This seems surprising but the micro distribution of prey species is worth further investigation.

The importance of self-generated gradients in long-range movement may be a more likely explanation for why we observed no learning. Chemotactic gradients established solely by the diffusion of attractants from a local source are only effective at guiding directional movement of cells over short distances (500 μm) (Tweedy et al. 2016). The gradient might be too shallow to establish any directional movement over large distances. At long distances (5 mm) self-generated gradients play an important role (Tweedy et al. 2016). Self-generated gradients occur when cells produce enzymes that break down the chemoattractant locally. This can steepen the attractant gradient and allow directional movement towards the source. *D. discoideum* amoebas secrete deaminases to break down folate (Bernstein et al. 1981), and cAMP phosphodiesterase to breakdown cAMP (Shaulsky et al. 1998). Our experiments

involve distances well above 500 μm, so attractant breakdown is likely to be quite important. Amoeba movement in this context might be limited to chemoattractants that can be degraded such as folate and cAMP. In experiments using much shorter distances, perhaps using microfluidic technology, amoebas may be able to respond to, and perhaps learn from, chemoattractants that they cannot degrade.

In conclusion, our study shows that innate preferences are a key component of foraging behavior in a microbial predator, *Dictyostelium discoideum*. We found no evidence for learned prey attraction behaviors. This is a puzzling result that merits further exploration. Future work could focus on the microstructure of bacterial soil communities experienced by amoebas and if that is conducive for learning. Whether or not the amoebas possess a flexible mechanism for in sensing different chemoattractants could be explored by study of their GPCR's. Finally, microfluidics experiments might uncover learned foraging behaviors at smaller scales that we tested.

Acknowledgements

This material is based upon work supported by the National Science Foundation (DEB 1753743 and DEB 2237266). We thank Debra Brock for inputs during the development of the project. We would also like to thank members of the Queller-Strassmann lab for useful discussions.

References:

Amacker, N., Z. Gao, J. Hu, A. L. C. Jousset, G. A. Kowalchuk, and S. Geisen. 2022. Protist feeding patterns and growth rate are related to their predatory impacts on soil bacterial communities. FEMS Microbiology Ecology 98:fiac057.

Artemenko, Y., T. J. Lampert, and P. N. Devreotes. 2014. Moving towards a paradigm: common mechanisms of chemotactic signaling in Dictyostelium and mammalian leukocytes. Cell. Mol. Life Sci. 71:3711–3747.

Bernays, E. A. 2000. Neural limitations in phytophagous insects: Implications for diet breadth and evolution of host affiliation. Annual Review of Entomology 27.

Bernstein, R. L., M. Tabler, D. Vestweber, and R. Van Driel. 1981. Extracellular folate deaminase of *Dictyostelium discoideum*. Biochimica et Biophysica Acta (BBA) - General Subjects 677:295–302.

Brock, D. A., T. S. Haselkorn, J. R. Garcia, U. Bashir, T. E. Douglas, J. Galloway, F. Brodie, D. C. Queller, and J. E. Strassmann. 2018. Diversity of free-living environmental bacteria and their interactions with a bactivorous amoeba. Front. Cell. Infect. Microbiol. 8:411.

Carrasco-Pujante, J., C. Bringas, I. Malaina, M. Fedetz, L. Martínez, G. Pérez-Yarza, M. Dolores Boyano, M. Berdieva, A. Goodkov, J. I. López, S. Knafo, and I. M. De la Fuente. 2021. Associative Conditioning Is a Robust Systemic Behavior in Unicellular Organisms: An Interspecies Comparison. Front Microbiol 12:707086.

Dukas, R. 2008. Evolutionary Biology of Insect Learning. Annual Review of Entomology 53:145–160. Annual Reviews.

Fortunato, A., J. E. Strassmann, L. Santorelli, and D. C. Queller. 2003. Co-occurrence in nature of different clones of the social amoeba, Dictyostelium discoideum. Molecular Ecology 12:1031–1038.

Gans, J., M. Wolinsky, and J. Dunbar. 2005. Computational Improvements Reveal Great Bacterial Diversity and High Metal Toxicity in Soil. Science 309:1387–1390. American Association for the Advancement of Science.

Gershman, S. J., P. E. Balbi, C. R. Gallistel, and J. Gunawardena. 2021. Reconsidering the evidence for learning in single cells. eLife 10:e61907. eLife Sciences Publications, Ltd.

Giurfa, M., J. N►►ez, L. Chittka, and R. Menzel. 1995. Colour preferences of flower-naive honeybees. J Comp Physiol A 177.

Guilford. 1990. The evolution of aposematism. P. *in* Insect defences: adaptive mechanisms and strategies of prey and predators. Suny Press.

Hadfield, J. D. 2010. MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. Journal of Statistical Software 33:1–22.

Heidel, A. J., H. M. Lawal, M. Felder, C. Schilde, N. R. Helps, B. Tunggal, F. Rivero, U. John, M. Schleicher, L. Eichinger, M. Platzer, A. A. Noegel, P. Schaap, and G. Glockner. 2011. Phylogeny-wide analysis of social amoeba genomes highlights ancient origins for complex intercellular communication. Genome Research 21:1882–1891.

Johnke, J., Y. Cohen, M. de Leeuw, A. Kushmaro, E. Jurkevitch, and A. Chatzinotas. 2014. Multiple micro-predators controlling bacterial communities in the environment. Current Opinion in Biotechnology 27:185–190.

Jousset, A. 2012. Ecological and evolutive implications of bacterial defences against predators: antipredator defences and bacterial ecology. Environmental Microbiology 14:1830–1843.

King, J. S., and R. H. Insall. 2009. Chemotaxis: finding the way forward with Dictyostelium. Trends in Cell Biology 19:523–530.

Klein, P. S., T. J. Sun, C. L. Saxe, A. R. Kimmel, R. L. Johnson, and P. N. Devreotes. 1988. A Chemoattractant Receptor Controls Development in Dictyostelium discoideum. Science 241:1467–1472. American Association for the Advancement of Science.

Lenth, R. V., P. Buerkner, M. Herve, M. Jung, J. Love, and F. Miguez. 2022. Emmeans: Estimated Marginal Means, aka Least-Squares Means, Version 1.8. 1-1.

Letunic, I., and P. Bork. 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Research 49:W293–W296.

Meena, N. P., and A. R. Kimmel. 2017. Chemotactic network responses to live bacteria show independence of phagocytosis from chemoreceptor sensing. eLife 6:e24627. eLife Sciences Publications, Ltd.

Mery, F., and J. G. Burns. 2010. Behavioural plasticity: an interaction between evolution and experience. Evol Ecol 24:571–583.

Minh, B. Q., H. A. Schmidt, O. Chernomor, D. Schrempf, M. D. Woodhams, A. von Haeseler, and R. Lanfear. 2020. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Molecular Biology and Evolution 37:1530–1534.

Mishra, A., A. Jain, P. S. Iyer, A. Suryanarayanan, K. Nordström, and S. B. Olsson. 2024. Innate floral object identification in the generalist solitary hoverfly pollinator *Eristalinus aeneus*.

Nakagawa, S., and H. Schielzeth. 2013. A general and simple method for obtaining R^2 from generalized linear mixed-effects models. Methods Ecol Evol 4:133–142.

Pan, M., X. Xu, Y. Chen, and T. Jin. 2016. Identification of a Chemoattractant G-Protein-Coupled Receptor for Folic Acid that Controls Both Chemotaxis and Phagocytosis. Developmental Cell 36:428–439.

Pan, P., E. M. Hall, and J. T. Bonner. 1972. Folic Acid as Second Chemotactic Substance in the Cellular Slime Moulds. Nature New Biology 237:181–182. Nature Publishing Group.

Paradis, E., and K. Schliep. 2019. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35:526–528.

Queller, D. C. 2014. Joint phenotypes, evolutionary conflict and the fundamental theorem of natural selection. Philosophical Transactions of the Royal Society B: Biological Sciences 369:20130423. Royal Society.

Rashidi, G., and E. A. Ostrowski. 2019. Phagocyte chase behaviours: discrimination between Gram-negative and Gram-positive bacteria by amoebae. Biol. Lett. 15:20180607.

Reid, C. R. 2023. Thoughts from the forest floor: a review of cognition in the slime mould Physarum polycephalum. Anim Cogn 26:1783–1797.

Schloss, P. D., and J. Handelsman. 2006. Toward a Census of Bacteria in Soil. PLOS Computational Biology 2:e92. Public Library of Science.

Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675. Nature Publishing Group.

Shaulsky, G., D. Fuller, and W. F. Loomis. 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development 125:691–699.

Sherr, E. B., and B. F. Sherr. 2002. Significance of predation by protists in aquatic microbial food webs. Antonie van Leeuwenhoek 81:16.

Shreenidhi, P. M., D. A. Brock, R. I. McCabe, J. E. Strassmann, and D. C. Queller. 2024. Costs of being a diet generalist for the protist predator *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. 121:e2313203121.

Smith, W. P. J., B. R. Wucher, C. D. Nadell, and K. R. Foster. 2023. Bacterial defences: mechanisms, evolution and antimicrobial resistance. Nat Rev Microbiol 21:519–534.

Steele, M. I., J. M. Peiser, P. M. Shreenidhi, J. E. Strassmann, and D. C. Queller. 2023. Predation-resistant *Pseudomonas* bacteria engage in symbiont-like behavior with the social amoeba *Dictyostelium discoideum*. The ISME Journal 17:2352–2361.

Stephens, D. W. 1991. Change, regularity, and value in the evolution of animal learning. Behavioral Ecology 2:77–89.

Strassmann, J. E., and D. C. Queller. 2011. Evolution of cooperation and control of cheating in a social microbe. Proceedings of the National Academy of Sciences 108:10855–10862.

Tweedy, L., D. A. Knecht, G. M. Mackay, and R. H. Insall. 2016. Self-Generated Chemoattractant Gradients: Attractant Depletion Extends the Range and Robustness of Chemotaxis. PLOS Biology 14:e1002404. Public Library of Science.

Vos, M., A. B. Wolf, S. J. Jennings, and G. A. Kowalchuk. 2013. Micro-scale determinants of bacterial diversity in soil. FEMS Microbiol Rev 37:936–954.

Figures:

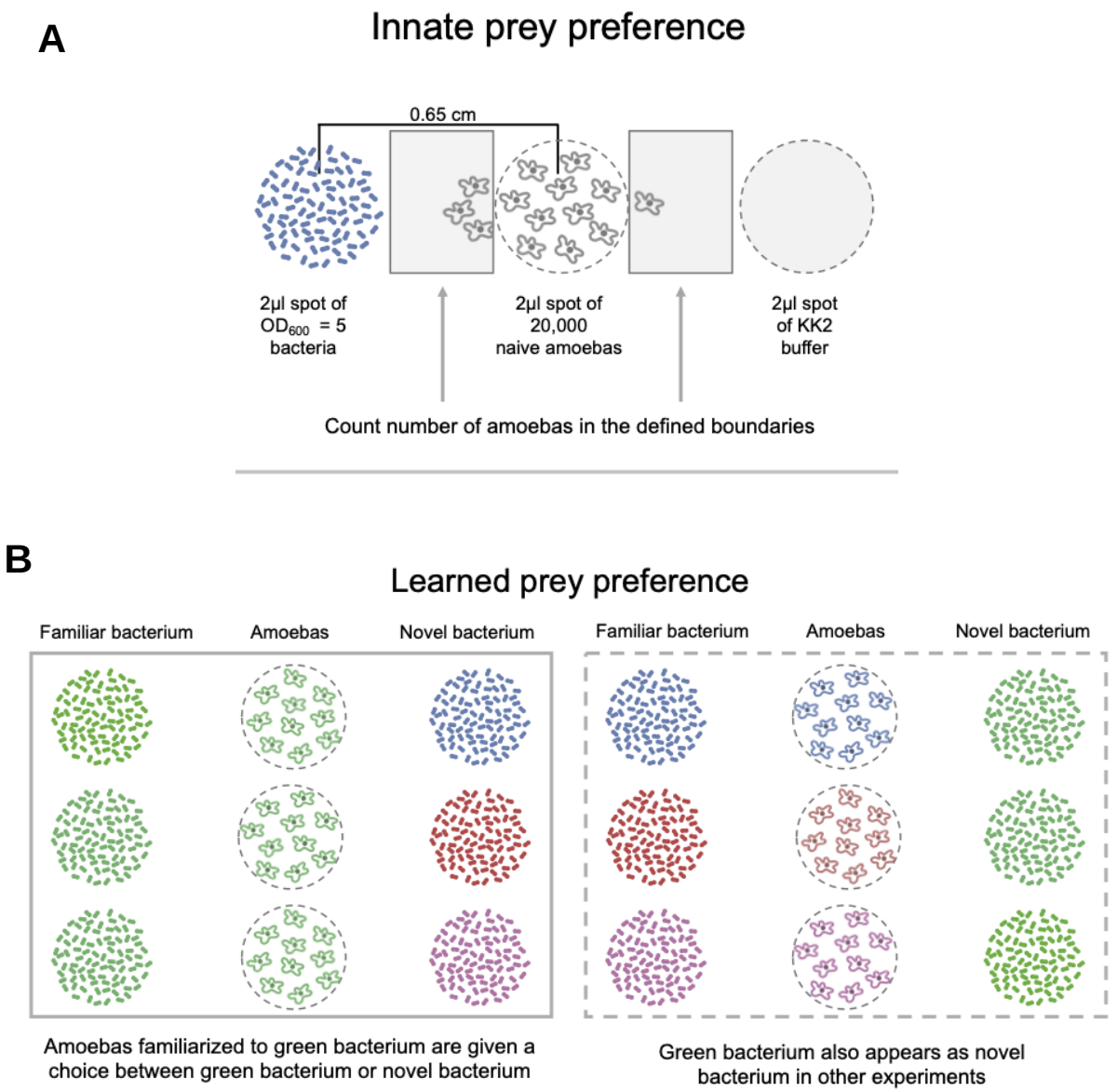

Figure 1.Experimental set-up. A) Innate prey preference in *D. discoideum* amoebas: A spot of AX4 amoebas was placed between spots of bacteria and KK2 buffer. The amoeba spot was imaged after 6 hours to estimate the number of amoebas that move towards the bacteria and buffer. **B) Learned prey preference in *D. discoideum* amoebas**: QS9 amoebas grown on bacterium A (referred to as familiar bacterium) were given a choice between a familiar and a novel bacterium. The number of amoebas that move towards the familiar and novel bacterium were counted at 4 h and 6 h. Each bacterium appears as both familiar and

novel in these experiments. Though we represented only three bacterial species for this experiment, the full design included fully factorial tests with eight species.

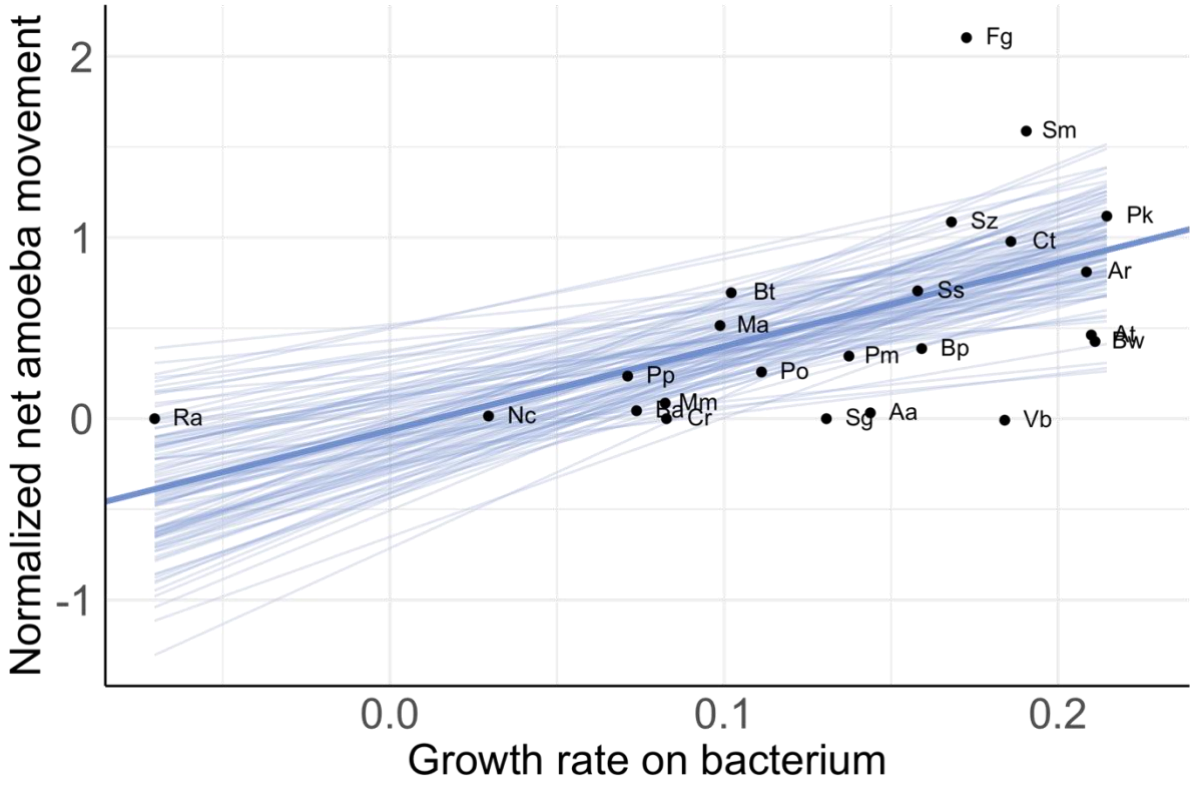

Figure 2. Amoebas are innately attracted to more profitable prey bacterium. Normalized movement of naïve amoebas towards different species of bacteria increases with increasing growth rate of amoebas on these bacteria (MCMCglmm, Fixed effect posterior mean = 4.58, 95% CI (1.00, 8.11), pMCMC = 0.011, R^2 = 0.21). Each point is an average of three biological replicates of amoeba growth rate and three technical replicates of amoeba movement on 23 species of soil bacteria. The solid blue line represents the mean value of slope and intercept from the MCMC glmm model. The fine blue lines are a sampling of 100 slopes and intercepts that lie within the 95% CI distribution of these values from the model.

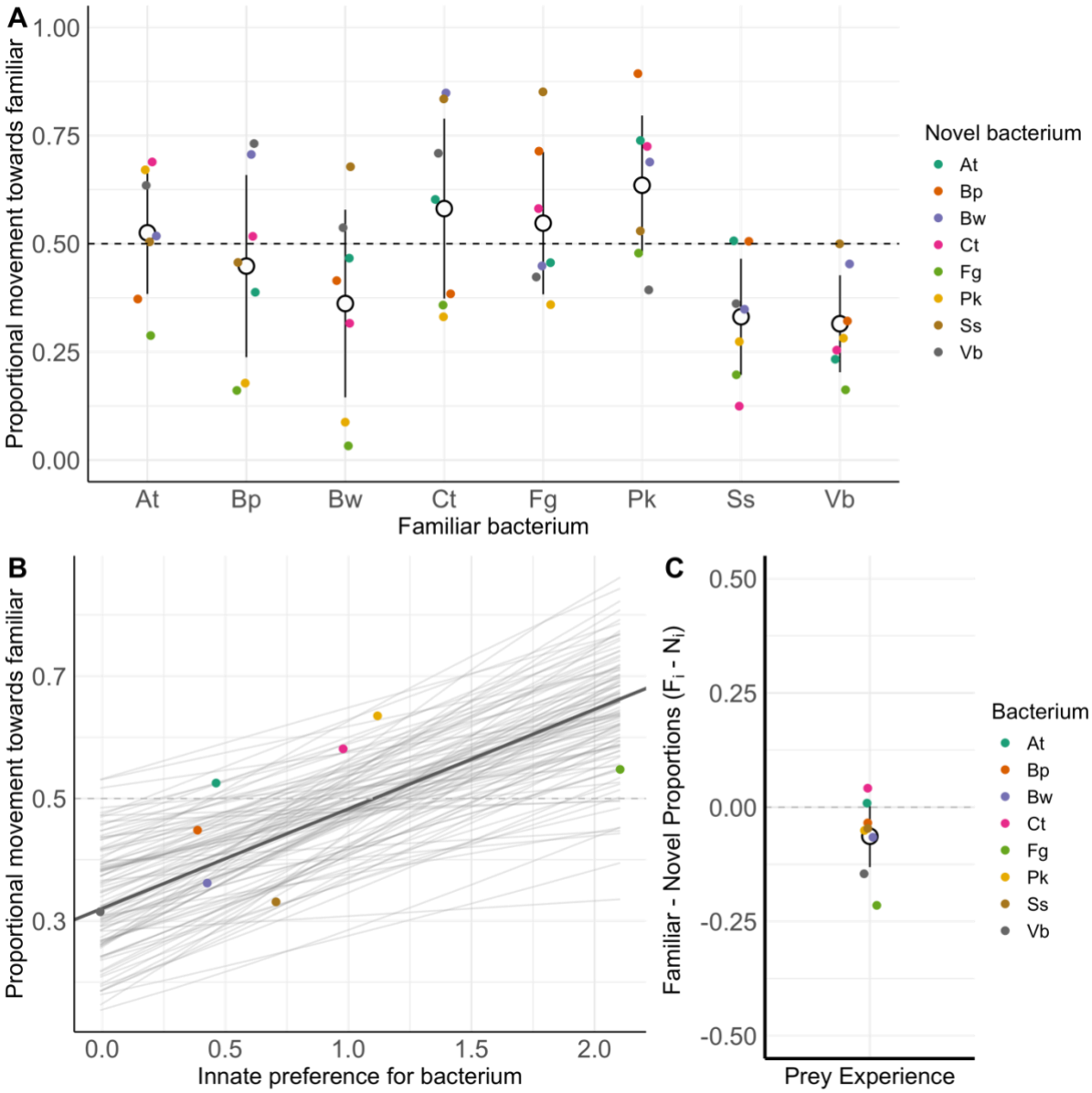

Figure 3. A) Amoebas varied in the level of attraction towards the familiar bacterium. Proportional movement of amoebas towards familiar bacterium averaged across 7 species of novel bacteria. The white circle and error bars mark the mean and 95% confidence interval. **B) The variation in preference for the familiar bacterium was positively correlated with the innate preference for the bacterium** (MCMCglmm, Fixed effect posterior mean = 0.16, 95% CI (-0.02, 0.36), pMCMC = 0.07, R^2 = 0.28). **C) Prey experience does not modulate prey preference in *D. discoideum* amoebas.** Difference in proportional movement of amoebas when they are familiar compared to when they are novel, averaged across all partner bacterial species. There was no significant difference in average movement of amoebas when

bacteria are familiar compared to when bacteria are novel. The white circle with the error bars marks the mean plus 95% confidence intervals.

Table 1: List of all bacteria used in innate preference experiments. The bacteria marked in green were used in learned preference experiments.

Strain number	Closest species by 16s (Brock2018)	Abbreviation	Phylum/Class	Gram status
18P 8.1.1	*Achromobacter aegrifaciens*	Aa	Betaproteobacteria	Negative
20P 9.1.2	*Agrobacterium rubi*	Ar	Alphaproteobacteria	Negative
18P 2.2.1	*Agrobacterium tumefaciens*	At	Alphaproteobacteria	Negative
20P 7.2.1	*Bacillus aryabhattai*	Ba	Firmicutes	Positive
20P 6.1.2	*Brucella papionis*	Bp	Alphaproteobacteria	Negative
17D 9.2.1	*Bacillus toyonensis*	Bt	Firmicutes	Positive
18P 8.2.2	*Buttiauxella warmboldiae*	Bw	Gammaproteobacteria	Negative
14P 4.3.2	*Chryseobacterium rhizosphaerae*	Cr	Bacteroidetes	Negative
18P 6.2.3	*Comamonas testosteroni*	Ct	Betaproteobacteria	Negative
20P 3.2.2	*Flavobacterium ginsengisoli*	Fg	Bacteroidetes	Negative
Lab clone	*Klebsiella pneumoniae*	Kp	Gammaproteobacteria	Negative
11P 7.1.1	*Mycobacterium arabiense*	Ma	Actinobacteria	Positive
14P 6.2.1	*Microbacterium maritypicum*	Mm	Actinobacteria	Positive
18P 5.1.3	*Nocardia coeliaca*	Nc	Actinobacteria	Positive
14P 8.1.1	*Pseudomonas kuykendallii*	Pk	Gammaproteobacteria	Negative
14P 5.3.2	*Pseudomonas migulae*	Pm	Gammaproteobacteria	Negative
20P 2.1.2	*Pandoraea oxalativorans*	Po	Betaproteobacteria	Negative
20P 2.1.1	*Paenibacillus pabuli*	Pp	Firmicutes	Positive
17P 9.1.1	*Rhodococcus agglutinans*	Ra	Actinobacteria	Positive
20P 10.2.1	*Sphingobacterium ginsensidimutans*	Sg	Bacteroidetes	Negative
14P 6.2.3	*Stenotrophomonas maltophilia*	Sm	Gammaproteobacteria	Negative
5S 2.1.1	*Staphlycoccus saprophyticus*	Ss	Firmicutes	Positive
20P 9.1.1	*Shinella zoogloeoides*	Sz	Alphaproteobacteria	Negative
20P 10.2.4	*Variovorax boronicumulans*	Vb	Betaproteobacteria	Negative

Supplementary figures:

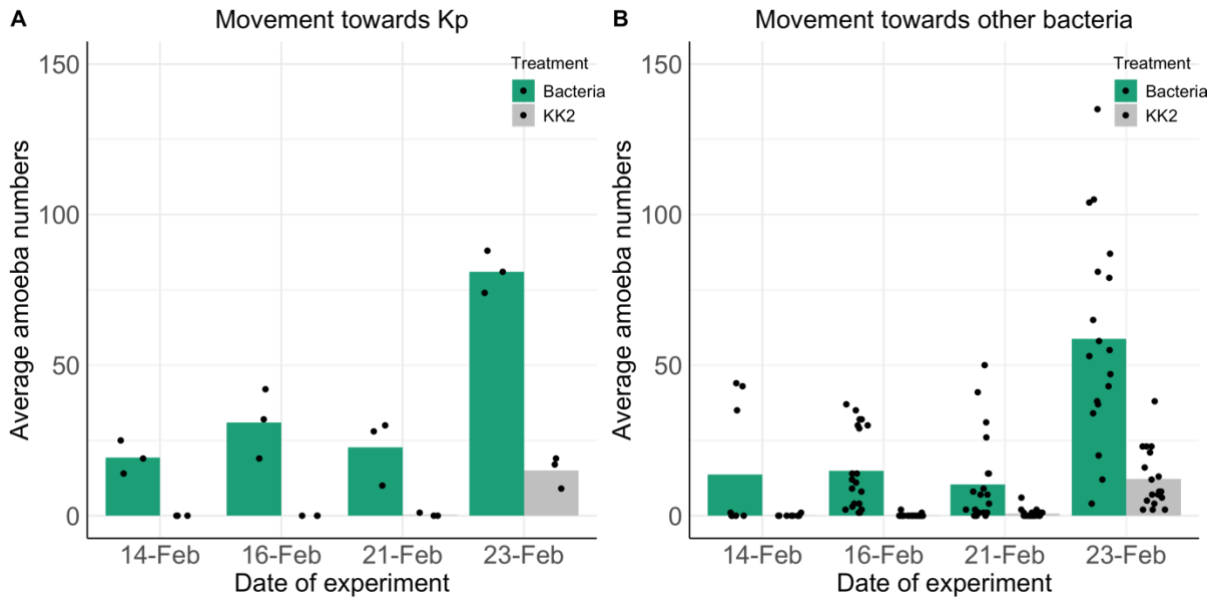

Figure S1: A) Movement of AX4 amoebas towards *Klebsiella penumoniae* on different days. B) Movement towards other bacteria on different days. Since chemotaxis experiments towards *Klebisella penumoniae* was repeated on all days, we can see that there are day-specific effects that need to be corrected for.

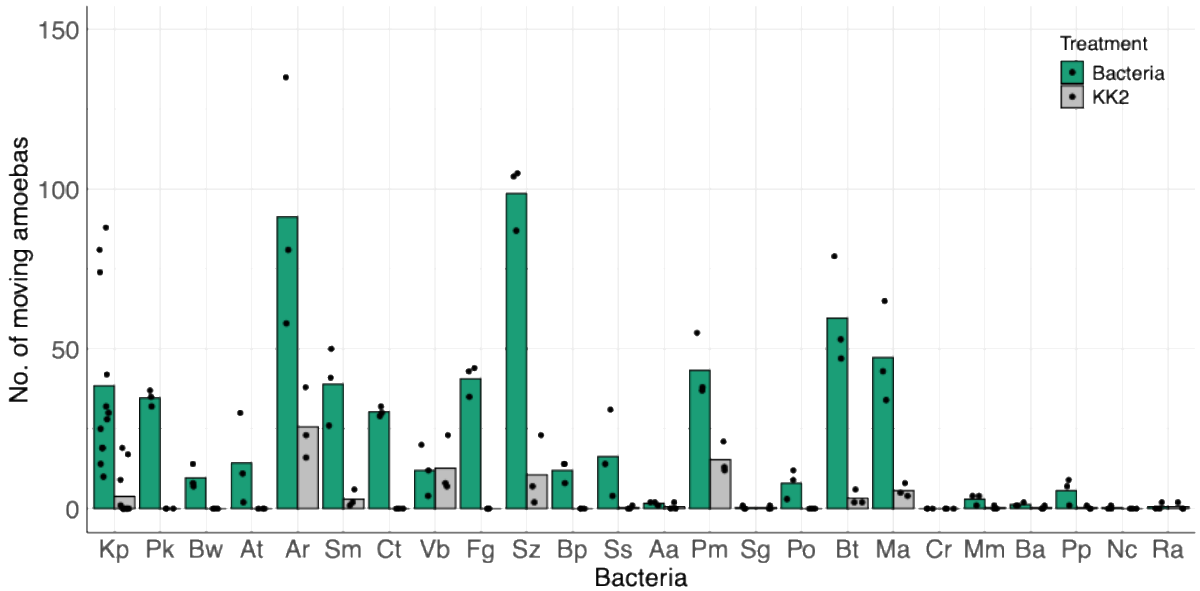

Figure S2: Data from innate preference experiments. The movement towards the bacterial spot is represented by green bars, and movement towards KK2 buffer is represented by grey bars. Amoebas generally move towards the bacteria and rarely to buffer. The bar marks the means across the 3 technical replicates (points).

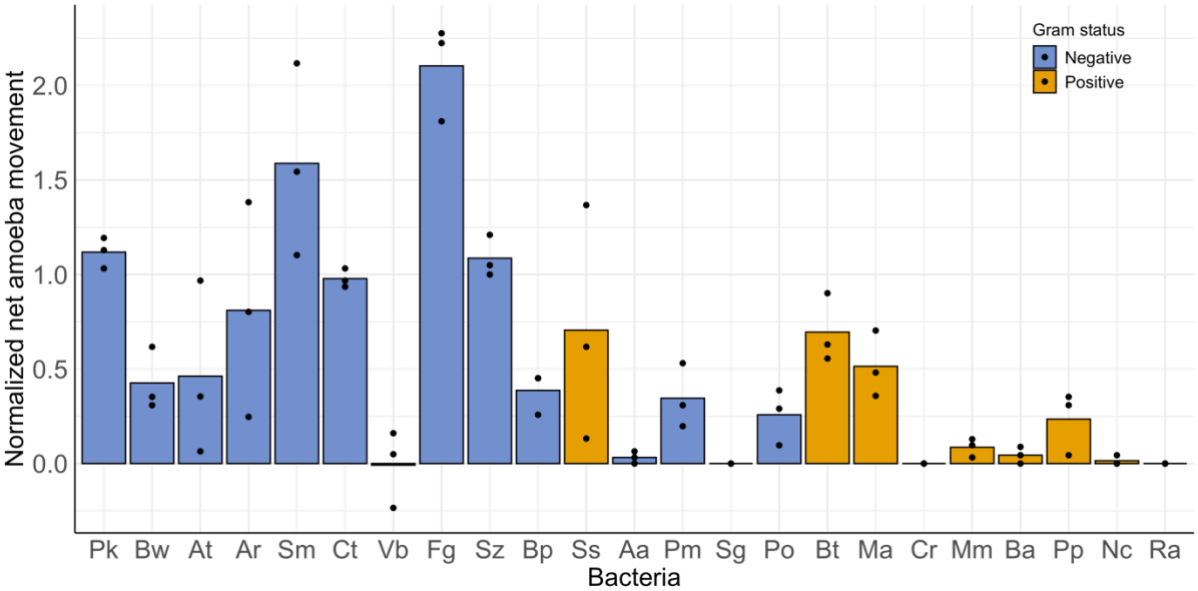

Figure S3: Normalized movement of AX4 amoebas towards different species of bacteria. We first calculated net number amoeba movement towards a bacterium in each replicate by subtracting number of amoebas that moved towards KK2. To make amoeba movement comparable across different days, we divided net movement towards bacterium by average net movement towards *Klebsiella pneumoniae* on that day. Bar marks the mean across 3 technical replicates (points).

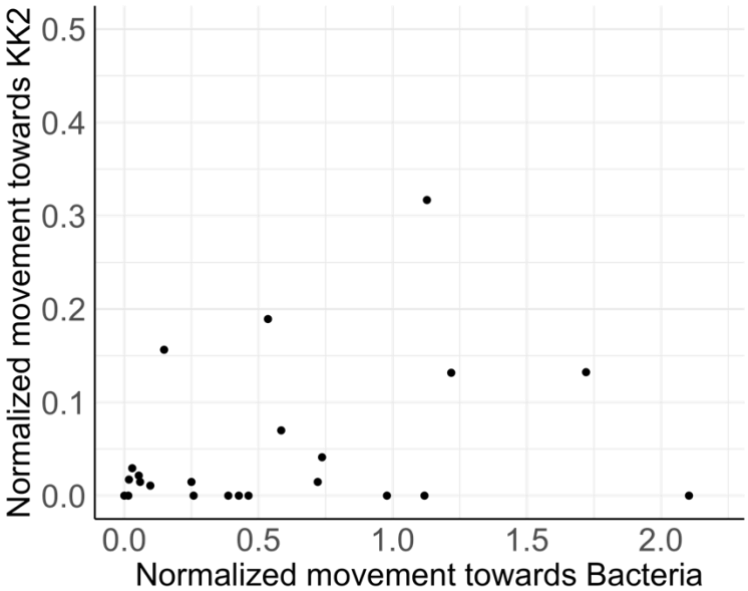

Figure S4: Normalized movement towards KK2 buffer Vs. Normalized movement towards bacteria. Here normalization means correction for any day effect, where we divided all movement on a given day by mean movement towards *Klebsiella pneumoniae* on that day. We found no significant relation between movement towards KK2 and movement towards bacteria.