Title
Interactions within Higher-order Antibiotic Combinations Influence the Rate of Adaptation in Bacteria

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Interactions within Higher-order Antibiotic Combinations Influence the Rate of Adaptation in Bacteria

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

Emoni Cook

2022

ABSTRACT OF THE THESIS

Interactions within Higher-order Antibiotic Combinations Influence the Rate of Adaptation in Bacteria

by

Emoni Cook

Master of Science in Biology

University of California, Los Angeles, 2022

Professor Pamela Yeh, Chair

Using multiple drugs in combination has been suggested as a possible solution to the antibiotic resistance problem. However, drug combinations introduce new factors to consider, including how the interactions among drugs influence the evolutionary process. Antibiotic combinations are considered additive if the combined effect is equivalent to the drugs acting independently, synergistic if more effective and antagonistic if less effective. This study examines the evolution of *S. epidermidis* in single drug, two-drug, and three-drug environments to determine how the interaction types may influence the rate of adaptation. The net interaction of a combination as well as the emergent interaction—the interaction that is uniquely due to all drugs being present in a combination that is not due to pairwise interactions, was examined. We find that in three-drug combinations, synergistic net interactions correlate with higher rates of adaptation, and the emergent interaction has no significant effect on the rates of adaptation.

The Thesis of Emoni Cook is approved.

Nandita Garud

Colin Kremer

Pamela Yeh, Chair

University California, Los Angeles

2022

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INTRODUCTION

Antibiotic-resistant bacteria are present in the majority of environments worldwide, occurring in both clinical and non-clinical settings (Esiobu et al., 2002; Martinez, 2009; Wright, 2010). Through the overuse and misuse of antibiotics, bacterial populations evolve resistance (Ventola, 2015). This creates an arms race between the development of new antibiotics and the evolution of resistance resulting in a global health crisis (Levy & Marshall, 2004; Andersson, 2006; Naik et al., 2022). While more bacteria continue to evolve single and multi-drug resistance (Bush et al., 2011; Spellberg & Gilbert, 2014), the discovery of new antibiotics has decreased dramatically (Nathan, 2004; Ventola, 2015). Antibiotic resistance has been projected to be responsible for 10 million deaths per year by 2050 if no new treatments or strategies are implemented (Tagliabue & Rappuoli, 2018). One solution to address this problem is to use antibiotics in combination (Fitzgerald et al., 2006; C. J. Brown et al., 2013; Foucquier & Guedj, 2015; E. D. Brown & Wright, 2016). However, combinations with multiple drugs introduce new factors to consider, including how the interactions between drugs influence the evolutionary process of antibiotic resistance.

When antibiotics are used in combination, the effects of the drugs themselves can interact with each other. These interactions can be categorized into three types: additive, synergistic, or antagonistic. An interaction is considered additive if the combination of drugs yields the same effect as if the single drugs were acting independently from each other (Bliss, 1939). A synergistic interaction gives a stronger response than expected; in the case of antibiotic interactions synergy would result in higher levels of growth inhibition (Bliss, 1939). *In vitro* studies have shown that these types of interactions can attain higher efficacy with lower concentrations however, they have been shown to promote the evolution of resistance (Hegreness et al., 2008; Michel et al., 2008). In contrast to synergistic interactions, antagonistic

interactions yield a weaker response (less inhibition of bacterial growth) than additive interactions (Bliss, 1939). Although higher concentrations of drugs within antagonistic combinations may be needed to obtain the desired degree of inhibition, they can also be more effective in preventing the evolution of resistance (Chait et al., 2007; Hegreness et al., 2008; Michel et al., 2008; Yeh et al., 2009).

The interactions of higher-order drug combinations (combinations that consist of three or more drugs) are more complex than the interaction of combinations consisting of only two drugs. This is because multiple interactions are occurring within a single higher-order combination. For example, in a three-drug combination, seven different factors contribute to the fitness effect of the combination. The first three factors are the effects of the three single antibiotics by themselves. The next three factors are the effects of the three pairwise interactions. The last factor is the effect of all three antibiotics interacting with each other. Thus, within a three-drug combination, there is a total of four interactions occurring simultaneously, the three pairwise interactions, and the interaction solely due to all three drugs being present (Beppler et al., 2016).

The interactions of a higher-order combination can be characterized in two ways: (1) the net interaction and (2) the emergent interaction. The net interaction of a higher-order combination is the overall effect of all possible interactions within the combination. The emergent interaction of a higher-order combination is the interaction that is due to all drugs being present and not a result of any lower-order interactions or single drug effects (Beppler et al., 2016). Most studies examine the overall fitness effects or *net* interactions of higher-ordered combinations (Zimmer et al., 2016; Katzir et al., 2019; Yilancioglu & Cokol, 2019); however, it is unknown if or how *emergent* interactions may influence the evolution of populations experiencing higher-order combinations of drugs. Emergent interactions have been found in

multidrug higher-order (three or more drugs) combinations (Beppler et al., 2016; Tekin et al., 2018). Understanding the properties of emergent interactions is crucial in having a complete picture of a complex system of stressors.

Here we examine how net and emergent interactions of three-drug combinations affect the rate of antibiotic resistance adaptation. The interactions of two-drug combinations have been examined extensively (Yeh et al., 2006; Yeh et al., 2009; K. Wood et al., 2012; K. B. Wood, 2016; Zimmer et al., 2016). It has been shown that they can influence the rates of resistance adaptations (Hegreness et al., 2008) and the likelihood of spontaneous resistance mutations (Michel et al., 2008). However, it is unclear how higher-order interactions affect the evolution of resistance. We ask the following questions: 1) Does the net interaction of three-drug combinations affect the rate of adaptation? 2) Does the emergent interaction of three-drug combinations affect the rates of adaptation?

MATERIALS AND METHODS

Bacterial strain and experimental evolution

We examined the evolution of *Staphylococcus epidermis* (ATCC 14990) populations to nine three-drug combinations (Table 1), all of the respective pairwise combinations (Table 1), and the single-drug treatments (Table 2). These drug combinations are comprised of a variety of antibiotics from different classes and have different main mechanisms of action (Table 2). For each drug treatment (three-drug combination, two-drug combination, and single drug) six populations were independently evolved. Each of these populations were evolved in one well on a 96-well plate with a working volume of 200 μL. For the first day of the experiment, plates were inoculated with cells via pin transferring (0.05 μL) of overnight cultures onto fresh plates. Plates were incubated at 37° C and had $O.D._{600nm}$ measurements taken every ten minutes for 23 hours

with a five-second orbital shake before each read. Populations were evolved over a fourteen-day period (roughly 150 generations). Every 24 hours, each population was pin-transferred (0.5 μL) over to a new plate containing fresh lysogeny broth media with the corresponding antibiotic treatment. Then, the new plate was incubated at 37° C and the $O.D._{600nm}$ measurements were taken every ten minutes for 23 hours with a five-second orbital shake before each read. The $O.D._{600nm}$ measurements taken on the first day of the experiment were used to determine the interaction values and fitness effects of the combinations. The $O.D._{600nm}$ measurements from all fourteen days were used to determine the rates of adaptation (see *Determination of Adaptation Rates* below).

Antibiotic Combinations and Interaction Values

The rescaled Bliss independence framework (RBI) (Tekin et al., 2016) was used to determine the interaction types and values of the combinations used here. For reference here is a brief overview of the framework. RBI uses Bliss independence (Bliss, 1939) as the additive model to evaluate interactions based on the relative fitness (w) to a no-drug control (Beppler et al., 2016; Tekin et al., 2016; Beppler et al., 2017; Tekin et al., 2017). Net interactions are determined using **Equation 1**. For example, in a two-drug combination, w_{AB} is the relative fitness of the bacterial population when treated with both drugs A and B in combination and $w_A w_B$ is the product of the relative fitnesses of being treated with drug A alone and drug B alone. If the deviation from additive effects (*DA/net*) is a positive value, it would indicate more growth than expected thus implying the interactions are antagonistic. A negative value would indicate a synergistic interaction.

$$\textbf{Equation 1: } \begin{cases} \textbf{two-drug combinations; } DA_{AB} = w_{AB} - w_A w_B \\ \textbf{three-drug combinations; } DA_{ABC} = w_{ABC} - w_A w_B w_C \end{cases}$$

Effectively the net interaction / deviation from additivity removes the additive effects of each individual drug. To find the emergent interactions we now must remove all the lower-order interactions from the DA leaving the value of the highest order interaction possible. To do this we used **Equation 2** which can all be expressed in terms of relative fitness resulting in **Equation 3**.

$$\textbf{Equation 2: } E3 = DA_{ABC} - DA_{AB}w_C - DA_{AC}w_B - DA_{BC}w_A$$

$$\textbf{Equation 3: } E3 = w_{ABC} - w_{AB}w_C - w_{AC}w_B - w_{BC}w_A + 2\,w_A w_B w_C$$

Then the net (*DA*) and emergent (*E3*) interactions were rescaled to enhance the ability to identify interactions occurring. They were normalized to either the lethal cases (when evaluating synergistic interactions) or to the most effective single or a subset of drugs (when evaluating additive and antagonistic interactions). When rescaling non-synergistic emergent interactions, we normalized to relative effects from pairwise interactions. For more details on the rational and exact equations used please refer to Tekin et al. (2016).

Determination of Adaptation Rates

Rates of adaptation were determined by the change in the growth term (*GT*) (defined in **Equation 4**) over time following similar methods to Hegreness et al. (2008). The growth term used is a function of the growth rate (*r*) and the time it takes to grow to half of the carrying capacity (*K).* We will refer to this as t_{mid}. This growth term was used to incorporate not only adaptations that increased growth rates but to also account for adaptations that reduced lag time promoting active growth in environments where antibiotics are subjugated to potential degradation (Li et al., 2016).

Equation 4: $$GT = \frac{r}{t_{mid}}$$

The growth rate (r), carrying capacity (K), and t_{mid} were all determined by fitting **Equation 5** to the OD data over time with the use of the Growthcurver (0.3.1) package in R (Sprouffske & Wagner, 2016).

Equation 5: $$OD(t) = \frac{K}{1+\left(\frac{K-N_0}{N_0}\right)e^{-rt}}$$

The adaptation rate (α) is equal to the change between the initial and final growth term of a population (ΔGT) divided by the time to navigate to a different fitness increase (t_{adapt}), **Equation 6** (Hegreness et al., 2008).

Equation 6: $$\alpha = \frac{\Delta GT/2}{t_{\text{adapt}}}$$

RESULTS

Accounting for selection pressures

Before any comparisons between interactions (additive, synergistic and antagonistic) and rates of adaptation were made, we determined the effects a combination has on the fitness of a population and the rates of adaptation. A Pearson correlation test was performed to measure the relationship between the two-drug and three-drug combinations relative fitness and rates of adaption, showing a significant correlation ($R = 0.23$, $p = 0.0017$) (Figure 1). We then took the residuals of this correlation to determine if any type of interaction may influence the rate of adaptation. These residual values were used when comparing any subset of the data when looking for correlations (following similar approaches outlined in (Baltagi, 1998)) between rates of adaptation and interactions. All rates of adaptation have been corrected for this relationship.

Interactions and Adaptation Rates

To determine how the net interactions correlate with rates of adaption, we performed a Pearson correlation on the populations that evolved and did not go extinct. We first observed the pooled data set comprising populations evolved to two-drug combinations and populations evolved to three-drug combinations. We observed a significant negative correlation (R= -0.23, p = 0.002) (

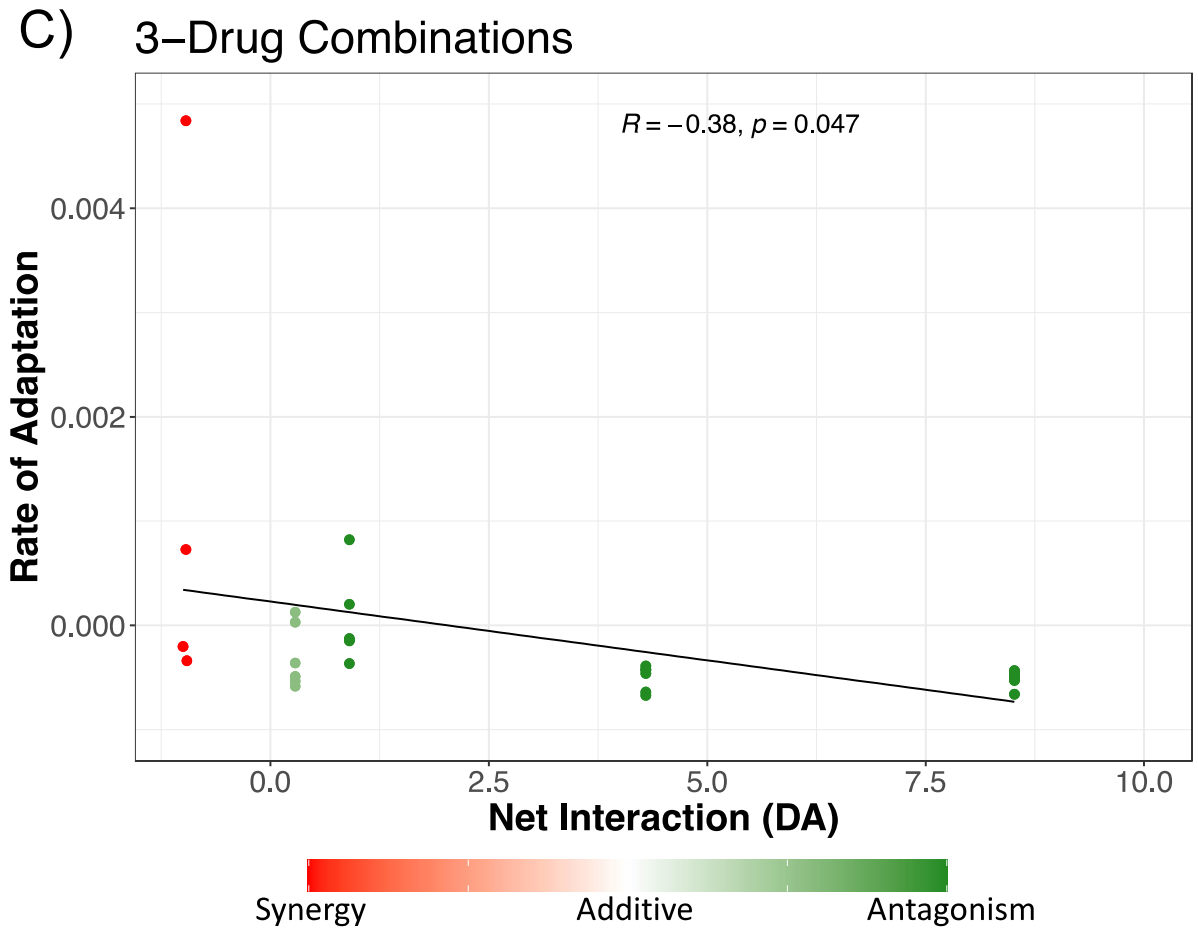


Figure 2A). As the net interaction values decrease and become more negative (synergistic) the rate of adaptation increases. We then examined if this relationship remained with the two-drug and the three-drug combinations separately. We found that the significant

negative correlation remained, but the degree of the correlation differed based on if there were two (R= -0.17, p = 0.037) or three (R= -0.38, p = 0.047) drugs in the combination (

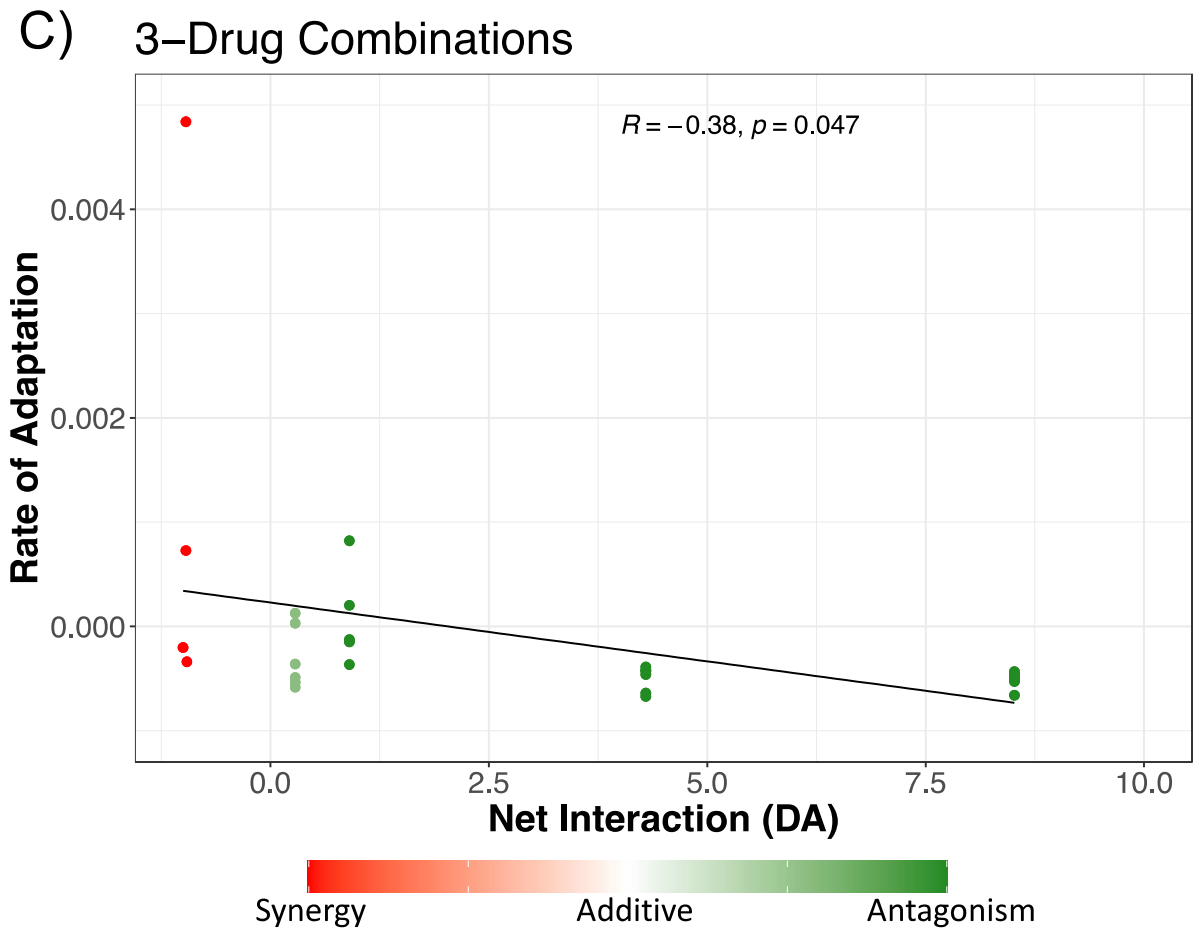


Figure **2**B and

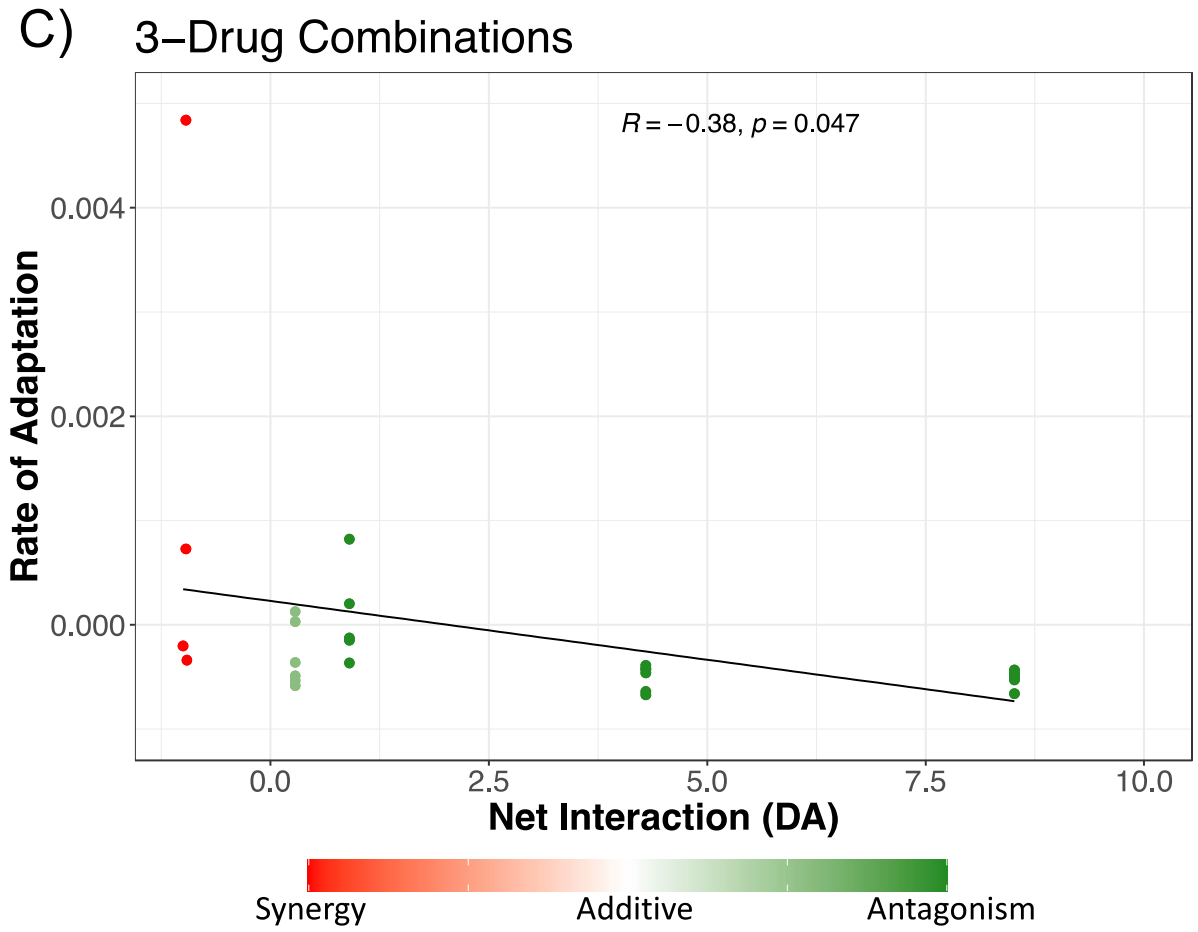


Figure **2**C respectively). We also asked if the emergent interactions of a three-drug combination correlate with rates of adaptation (Figure 3.). We performed another Pearson correlation test and found no significant correlation (R= 0.1, p = 0.6).

DISCUSSION

We asked how the interactions of a higher-order drug combination may correlate with the rates of adaption. We evolved multiple populations to a variety of three-drug combinations and all the corresponding two-drug and single-drug treatments over fourteen days. We found that the net interactions of both two-drug and three-drug combinations significantly correlate with the rates of adaptation, where more synergistic interactions correlated with faster rates of adaptation

(

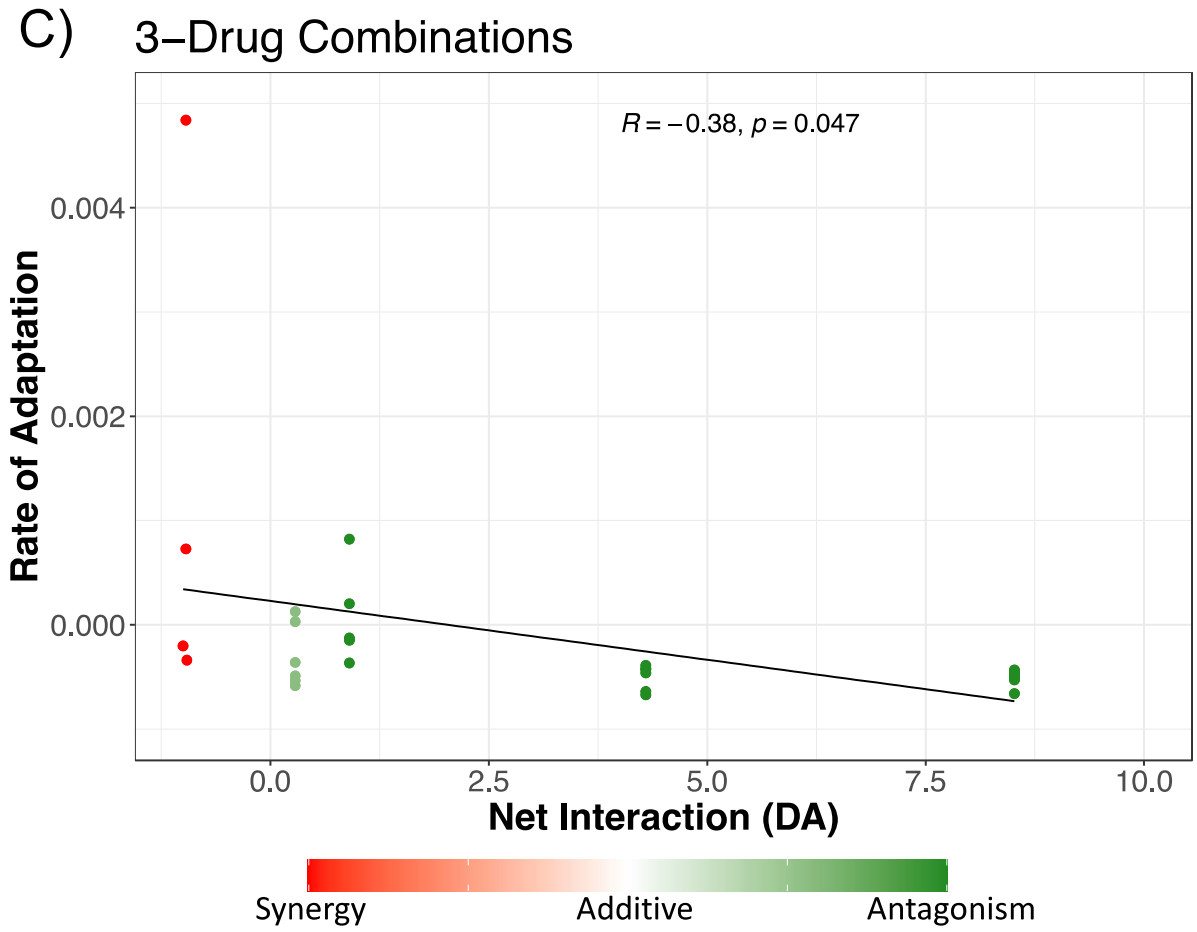


Figure 2). We also determined that the emergent interaction of a three-drug combination did not correlate with rates of adaptation (Figure 3.).

Hegreness et al. (2008) were the first to directly test if interactions between the antibiotics in a two-drug combination could correlate with adaptation rates. They evolved multiple populations of *Escherichia coli* to four different two-drug combinations for fifteen days (>150 generations). For each drug combination, a variety of doses and ratios were used and the interaction values were calculated for each drug-dose combination separately. This meant that two combinations with the same antibiotics but different doses could have different interaction values. Every 24 hours populations were transferred to fresh media that contained the same

antibiotic combination at the same dosage. Growth was measured and growth rates were determined to calculate adaptation rates. They found a significant positive correlation between the degree of synergy and rates of adaptation. That is combinations with synergistic interactions correlated with higher rates of adaptation.

Multiple studies in two-drug combinations have suggested synergistic interactions promote antibiotic resistance evolution and antagonistic interactions limit antibiotic resistance evolution. Synergistic interactions have a higher likelihood of spontaneous resistance mutations (Michel et al., 2008). In addition, synergistic interactions have been shown to select for resistance while antagonistic interactions do not select for resistance (Chait et al., 2007). This is especially true when bacterial populations are faced with competition. It has been suggested that initial treatment with synergistic drug combinations could result in a higher bacterial load after treatment when compared to initial treatment with additive or antagonistic combinations (Pena-Miller et al., 2013). Antagonistic drug pairs maintain competitive interactions between the wild-type and single-drug resistant populations thus limiting the growth and further mutation of the single-drug resistant bacteria. Antagonistic combinations can maintain this competition because they are effective at killing off the entire wild-type bacteria (Torella et al., 2010).

Some previous studies did not find significant correlations between drug interaction and rates of adaptions or evolvability and rather suggest that collateral effects between a pair of drugs affect the evolution of resistance. Collateral effects are the unintentional changes in phenotypic response to other stressors because of previous adaptations. When evaluating collateral effects of antibiotic resistance, evolving resistance to one antibiotic may result in either increased resistance (cross-resistance) or increased sensitivity (collateral sensitivity) to another antibiotic (Haight & Finland, 1952; Sanders, 2001; Obolski et al., 2015). In two-drug combinations,

mutations that confer drug resistance are typically not selected for if they also confer collateral sensitivity to the other drug in the combination, limiting antibiotic resistance adaptation within a population (Munck et al., 2014). Pairwise drug combinations that are either cross-resistant or do not have collateral effects on each other have higher evolvability than those with collaterally sensitive drug pairs (Rodriguez de Evgrafov et al., 2015).

More recent studies have suggested alternative factors in addition to collateral effects that can influence rates of adaptation. Multiple populations of *Pseudomonas aeruginosa* were evolved to 38 different pairwise combinations (based on a range of drug interactions and collateral effects) to assess the antibiotic combination efficiency (ACE) (Barbosa et al., 2018). The ACE characterizes the ability of antibiotic combinations to limit bacteria survival and limit antibiotic resistance adaptation over time. Many of the drug combinations examined had synergistic interactions (24 interactions) but some had additive and antagonistic combinations (14 interactions each). By categorizing the ACE into two networks based on population extinction or adaption rates, they discovered that reduction in adaptation rates is driven by two factors: the adaptation to the component of a drug combination that has a stronger selection pressure alone and the specific collateral effect. There was no significant relationship between drug interactions and evolvability although they did observe instances that supported synergistic interactions selecting for resistance. In addition, synergistic combinations were the only combinations to experience extinctions despite having the same inhibitory levels as the other interaction types. Our current study supports these conclusions from Barbosa et al. (2018) regarding the importance antibiotic interactions can have on resistance evolution.

Another suggested factor that can influence rates of adaptation to a combination of antibiotics is the type of genetic response required for adapting resistance. The adapted genetic

responses to single and two-drug combinations in *E. coli* were categorized to evaluate how different genetic responses affect evolvability among different two-drug combinations (Jahn et al., 2021). The resistant mutation(s) selected by the drug combination was then compared to the mutation(s) selected for by each of the individual components alone. This resulted in four categories to describe the genetic response of adapting resistance to the combination. The four categories are as follows: 1) mutations conferring resistance to both drugs are the same and are selected by the combination; 2) mutations conferring resistance to both drugs individually are different and are selected by the combination; 3) mutations conferring resistance to both drugs individually are different but the combination only selects for one; or 4) mutations selected by combination are different than those selected by the individual drugs. Drug combinations that require novel mutations to gain resistance to the combination (category 4) limit the evolution of resistance compared to combinations where the mutations required for resistance are also selected by at least one of the components (categories 1, 2, and 3) (Jahn et al., 2021). When examining three-drug combinations the additional drug brings more complexities to evaluate by having to simultaneously consider three two-drug combinations. These additional complexities encountered when evaluating the genetic responses to a higher-order combination is similar to the additional complexities of determining the interactions of a higher-order combination. We hope that future studies will begin to examine the interplay between the genetic responses and the interactions of higher-order antibiotic combinations.

Most of the studies that did not conclude that interactions can influence rates of adaptation or evolvability evolved populations to a dynamic environment—that is the antibiotic concentrations of the combinations were increased as the populations evolved (Munck et al., 2014; Rodriguez de Evgrafov et al., 2015; Jahn et al., 2021). Even if the two antibiotics were

kept with the same ratio the change of dosage can change the strength or even type of the interaction (Berenbaum et al., 1983). This could mean that the populations being evolved may not have been adapting in response to the same interaction over the entire course of the experiments.

In contrast, Hegreness et al. (2008) used constant ratio and dosage of the drug combinations for the entirety of each evolution experiment. Hegreness et al. (2008) also tested a wide range of interactions from a single drug combination by choosing a variety of ratios and dosages. They found a correlation between interaction and rate of adaptation hold within the same combination of two drugs for all four drug combinations tested. Additionally, within the strongly synergistic combination tested (the combination of erythromycin and doxycycline) the highest rates of adaptation were found for dosages that have higher amounts of synergy. These rates are even higher than those of the populations evolving to the single drug components alone. In contrast, within the strongly antagonistic combination (ciprofloxacin and doxycycline) there was a decreased rate of adaptation compared to the single drug components alone. Our current findings further support the conclusion that net synergies increase the rate of adaptation to antibiotics.

But beyond that, this study is the first to directly test the relevance of emergent interactions regarding the evolution of a population. Studying emergent interactions has been historically difficult to do because it requires a large-scale data set with a full factorial design, where all possible subsets and individual factors are tested independently. But more recently, emergent interactions have been systematically measured and shown to be very frequent among antibiotic combinations (Tekin et al., 2018; Lozano-Huntelman et al., 2020) and ecological

stressors (Diamant et al., 2022). Future studies can help elucidate the role emergent properties play in the evolution of a suite of population traits.

Tables and Figures:

Table 1. The combinations and concentrations of antibiotics used in three-drug combinations.

Fitness is expressed by relative fitness to a no-drug control.

three-Drug Antibiotic Combination	Net Interactions (DA)	Emergent Interactions (E3)	Fitness	two-drug Antibiotic Combination	Net Interactions (DA)	Fitness
CLI+FUS+TMP	10+	-0.545	2.069	CLI- FUS	-0.062	0.981
				CLI- TMP	5.503	2.987
				TMP- FUS	1.233	0.565
NEO+PIP+TMP	-0.967	0.394	0.049	NEO-PIP	1.069	1.277
				NEO-TMP	-0.140	1.017
				TMP-PIP	-0.890	0.162
GEN+PIP+TMP	-0.956	1.076	0.085	GEN-PIP	6.026	1.542
				GEN-TMP	-1	0
				TMP-PIP	-1	0
CHL+GEN+TET	8.516	0.185	2.139	CHL-GEN	-0.116	0.667
				CHL-TET	6.706	1.569
				TET-GEN	4.008	1.788
FUS+NAL+TMP	0.904	-1	1.110	FUS-NAL	-0.082	1.457
				FUS-TMP	2.656	1.310
				TMP-NAL	3.063	1.187
CHL+DOX+NAL	0.284	-0.614	1.814	CHL-DOX	2.823	1.431
				CHL-NAL	1.754	1.589
				NAL-DOX	1.222	1.869
CHL+ERY+NAL	10+	10+	0.239	CHL-ERY	-0.045	1.499
				CHL-NAL	1	0
				NAL-ERY	1	0
FUS+OX+TET	-1	1	0.010	FUS-OX	-0.223	1.196
				FUS-TET	-1	0
				TET-OX	-1	0
FOX+GEN+TET	4.297	-0.133	1.987	FOX-GEN	-0.024	0.920
				FOX-TET	2.771	1.417
				TET-GEN	5.271	2.089

Table 2. The class and the main mechanism of action for the 12 antibiotics used in this study. Fitness is expressed by relative fitness to a no-drug control.

Antibiotic	Abbr.	Mechanism of Action	Class	Concentration (μMol)	Fitness
Cefoxitin sodium salt	FOX	Protein synthesis, 50S	Beta-lactam; Cephalosporins	0.7	0.948
Chloramphenicol	CHL	Protein synthesis, 50S	Broad-spectrum	90	1.054
Clindamycin hydrochloride	CLI	Protein synthesis, 50S	Macrolides	0.01	1.023
Doxycycline hyclate	DOX	Protein synthesis, 30S	Tetracyclines	0.6	1.215
Fusidic acid	FUS	Protein synthesis, 50S	Fusidane	0.005	1.043
Gentamicin sulfate	GEN	Protein synthesis, 50S	Aminoglycosides	0.15	0.965
Nalidixic acid sodium salt	NAL	DNA Gyrase	Quinolone	20	1.492
Neomycin	NEO	Protein synthesis, 50S	Aminoglycosides	0.35	0.893
Oxacillin sodium salt	OX	Cell Wall	Beta-lactam; Penicillin	0.005	1.193
Piperacillin sodium salt	PIP	Cell Wall	Beta-lactam: Penicillin	0.6	1.061
Tetracycline	TET	Protein synthesis, 30S	Tetracyclines	20	0.810
Erythromycin	ERY	Protein synthesis, 50S	Macrolides	0.05	1.070
Trimethoprim	TMP	Folic Acid	Antifolate	1.5	0.065

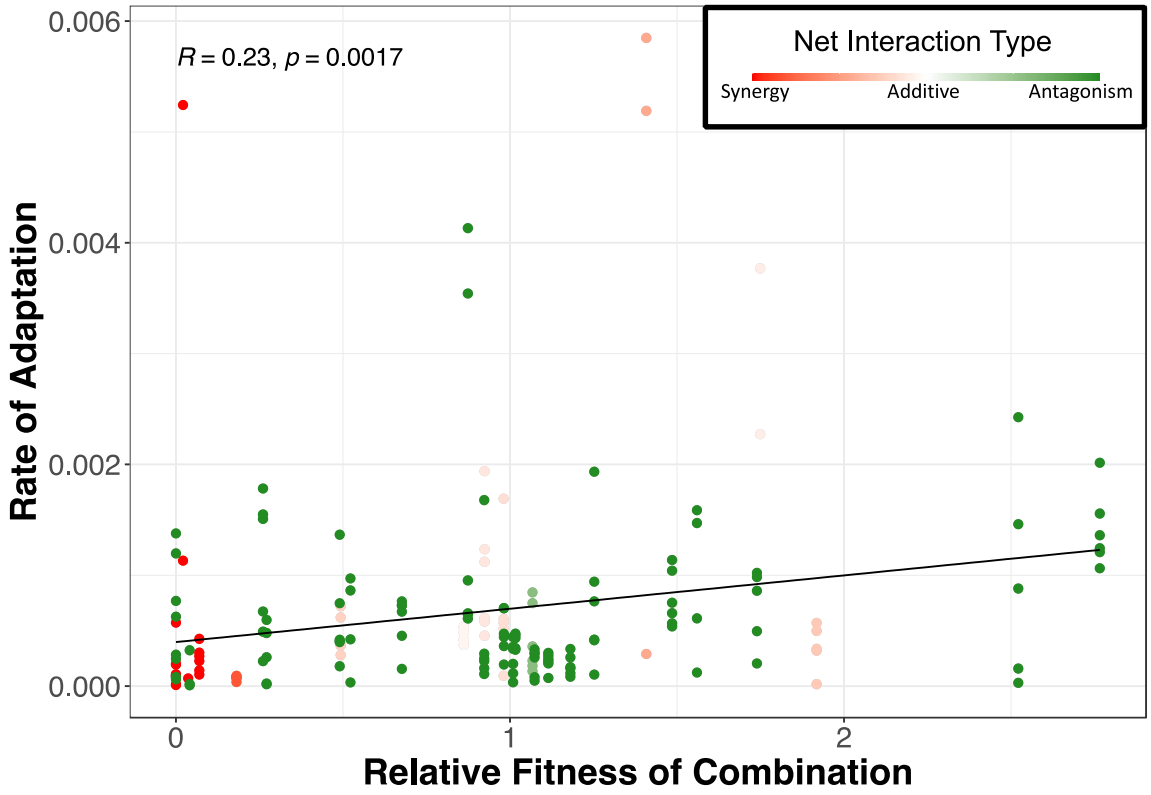
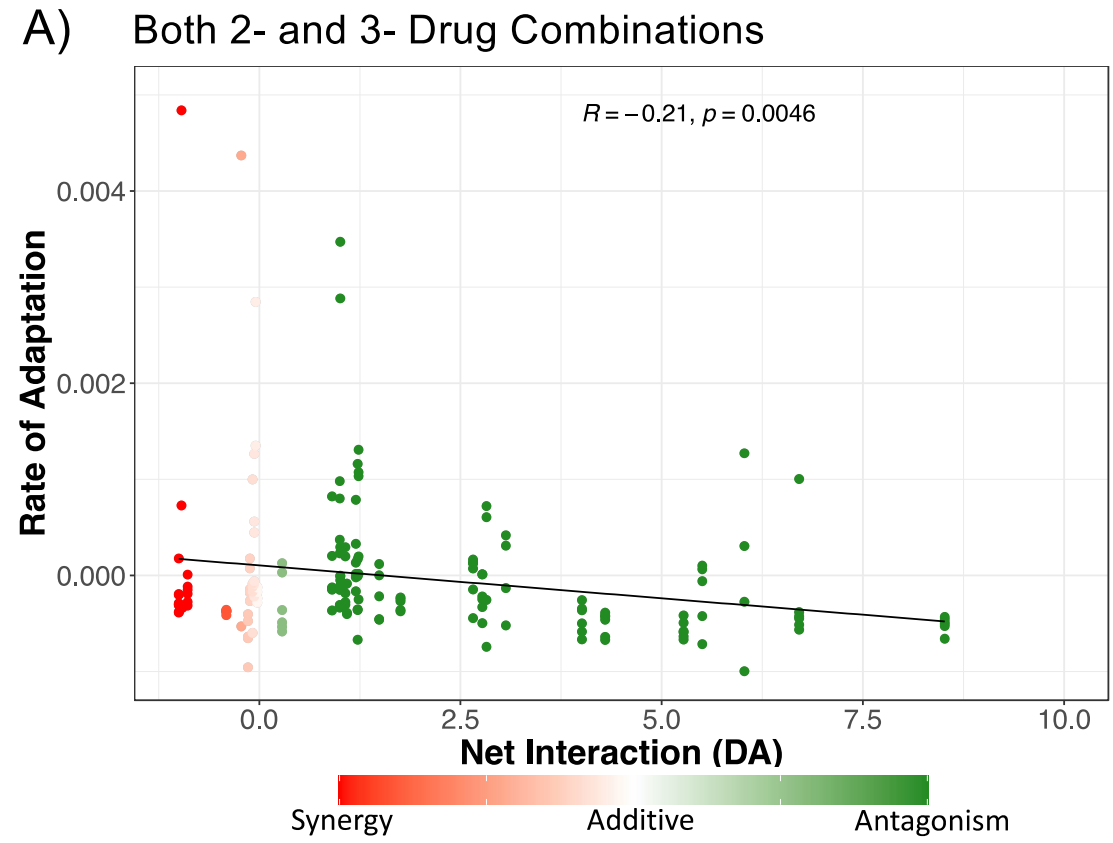
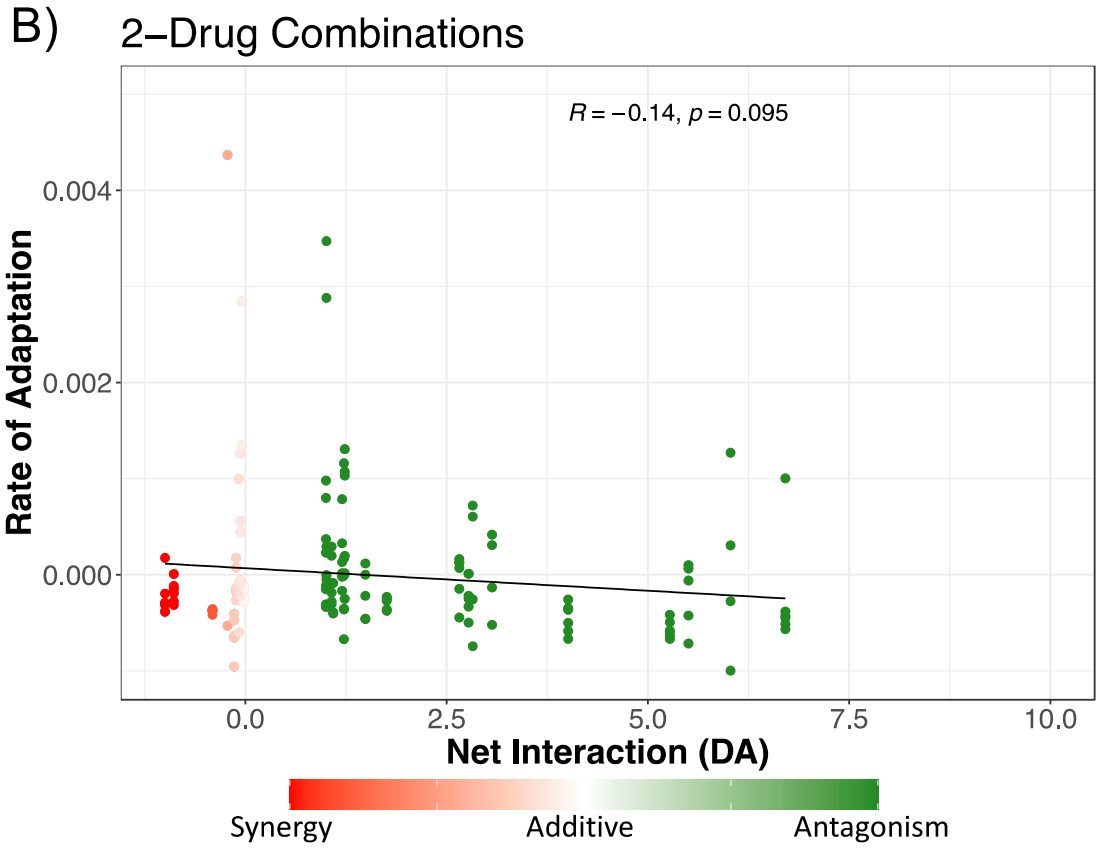


Figure 1. The correlation between relative fitness of the ancestral strain exposed to a combination and rate of adaptation. A Pearson correlation test was performed to measure the relationship between fitness and rates of adaption, showing a significant correlation (R = 0.23, p = 0.0017). The data from both two- and three- drug combinations were pooled together.


A)
Both 2- and 3- Drug Combinations
R = −0.21, p = 0.0046
Rate of Adaptation
0.004
0.002
0.000
0.0
2.5
5.0
7.5
10.0
Net Interaction (DA)
Synergy
Additive
Antagonism


B)
2–Drug Combinations
R = −0.14, p = 0.095
Rate of Adaptation
0.004
0.002
0.000
0.0
2.5
5.0
7.5
10.0
Net Interaction (DA)
Synergy
Additive
Antagonism

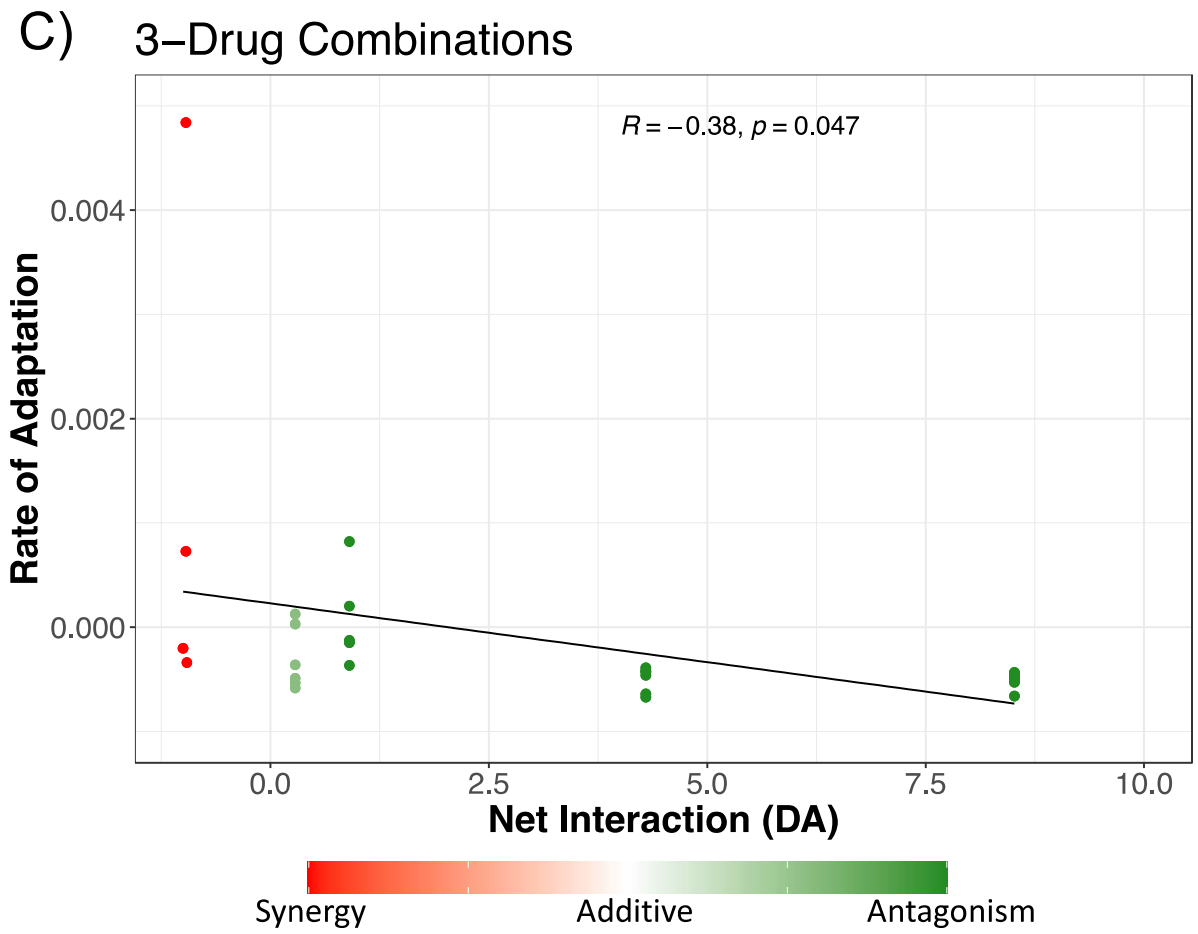


Figure 2. Synergistic combinations of two- and three- drugs correlate with faster rates of resistance adaptation. *A)* A Pearson correlation was performed on the corrected rates of adaptation and the net interaction of the pooled combination data for both two-drug and three-drug combinations. There was a significant negative correlation (R= -0.23, p = 0.002) which indicated that as net interactions become synergistic there are faster rates of adaptation. This trend is also observed when only examining *B)*, two- drug combinations (R= -0.17, p = 0.037) or *C)*, three-drug combinations separately (R= -0.38, p = 0.047).

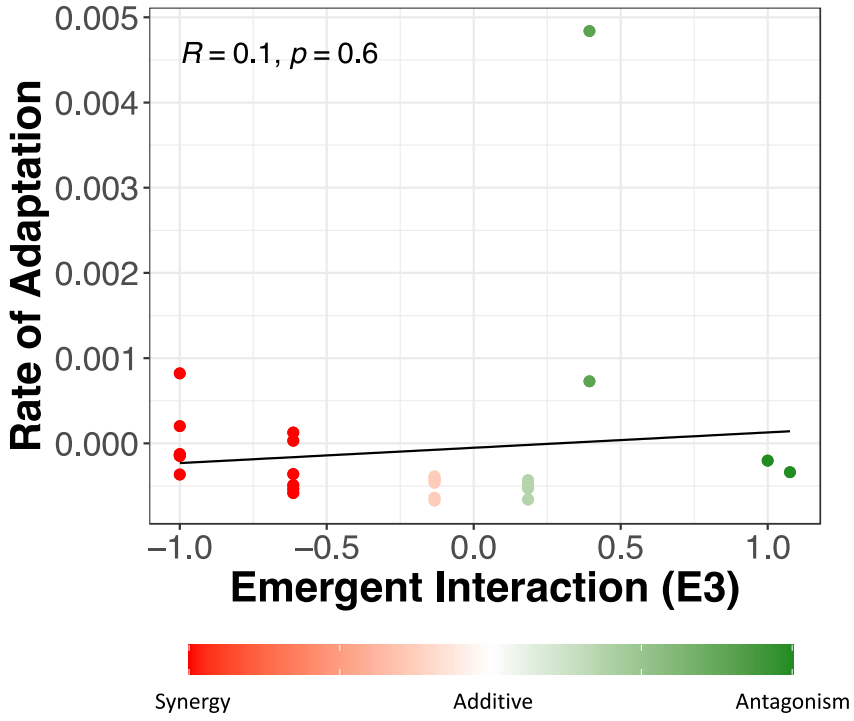


Figure 3 Emergent interactions do not correlate to rates of adaptation. A Pearson correlation was performed on the corrected rates of adaptation and the emergent interactions of the three-drug combinations. No significant correlation was found (R= 0.1, p= 0.6).

References

Andersson, D. I. (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Current opinion in microbiology*, *9*(5), 461-465. doi:0.1016/j.mib.2006.07.002

Barbosa, C., Beardmore, R., Schulenburg, H., & Jansen, G. (2018). Antibiotic combination efficacy (ACE) networks for a Pseudomonas aeruginosa model. *PLoS biology*, *16*(4), e2004356.

Beppler, C., Tekin, E., Mao, Z., White, C., McDiarmid, C., Vargas, E., . . . Yeh, P. J. (2016). Uncovering emergent interactions in three-way combinations of stressors. *Journal of The Royal Society Interface*, *13*(125), 20160800. doi:10.1098/rsif.2016.0800

Beppler, C., Tekin, E., White, C., Mao, Z., Miller, J. H., Damoiseaux, R., . . . Yeh, P. J. (2017). When more is less: Emergent suppressive interactions in three-drug combinations. *BMC microbiology*, *17*(1), 107. doi:10.1186/s12866-017-1017-3

Berenbaum, M. C., Yu, V. L., & Felegie, T. P. (1983). Synergy with double and triple antibiotic combinations compared. *Journal of Antimicrobial Chemotherapy, 12*(6), 555-563.

Bliss, C. (1939). The toxicity of poisons applied jointly. *Annals of Applied Biology, 26*(3), 585-615.

Brown, C. J., Saunders, M. I., Possingham, H. P., & Richardson, A. J. (2013). Managing for interactions between local and global stressors of ecosystems. *PLOS One, 8*(6), e65765.

Brown, E. D., & Wright, G. D. (2016). Antibacterial drug discovery in the resistance era. *Nature, 529*(7586), 336. doi:10.1038/nature17042

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., . . . Kutter, E. (2011). Tackling antibiotic resistance. *Nature Reviews Microbiology, 9*(12), 894.

Chait, R., Craney, A., & Kishony, R. (2007). Antibiotic interactions that select against resistance. *Nature*, *446*(7136), 668-671.

Diamant, E. S., Boyd, S., Lozano-Huntelman, N. A., Enriquez, V., Kim, A., Savage, V. M., & Yeh, P. (in prep). Re-analysis of three-stressor interactions reveal substantial higher-order interactions and emergent properties.

Esiobu, N., Armenta, L., & Ike, J. (2002). Antibiotic resistance in soil and water environments. *International Journal of Environmental Health Research*, *12*(2), 133-144.

Fitzgerald, J. B., Schoeberl, B., Nielsen, U. B., & Sorger, P. K. (2006). Systems biology and combination therapy in the quest for clinical efficacy. *Nature chemical biology*, *2*(9), 458-466.

Foucquier, J., & Guedj, M. (2015). Analysis of drug combinations: current methodological landscape. *Pharmacology research & perspectives, 3*(3), e00149.

Haight, T. H., & Finland, M. (1952). Resistance of bacteria to erythromycin. *Proceedings of the Society for Experimental Biology and Medicine, 81*(1), 183-188.

Hegreness, M., Shoresh, N., Damian, D., Hartl, D., & Kishony, R. (2008). Accelerated evolution of resistance in multidrug environments. *Proceedings of the National Academy of Sciences, 105*(37), 13977-13981. doi:10.1073/pnas.0805965105

Jahn, L. J., Simon, D., Jensen, M., Bradshaw, C., Ellabaan, M. M. H., & Sommer, M. O. A. (2021). Compatibility of evolutionary responses to constituent antibiotics drive resistance evolution to drug pairs. *Molecular biology and evolution, 38*(5), 2057-2069.

Katzir, I., Cokol, M., Aldridge, B. B., & Alon, U. (2019). Prediction of ultra-high-order antibiotic combinations based on pairwise interactions. *PLoS computational biology, 15*(1), e1006774.

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine, 10*(12s), S122.

Li, B., Qiu, Y., Shi, H., & Yin, H. (2016). The importance of lag time extension in determining bacterial resistance to antibiotics. *Analyst, 141*(10), 3059-3067.

Lozano-Huntelman, N. A., Zhou, A., & Yeh, P. (2020). Hidden suppressive interactions are common in higher-order drug combinations *iScience*.

Martinez, J. L. (2009). Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environmental pollution, 157*(11), 2893-2902.

Michel, J.-B., Yeh, P. J., Chait, R., Moellering, R. C., & Kishony, R. (2008). Drug interactions modulate the potential for evolution of resistance. *Proceedings of the National Academy of Sciences, 105*(39), 14918-14923. doi:10.1073/pnas.0800944105

Munck, C., Gumpert, H. K., Wallin, A. I. N., Wang, H. H., & Sommer, M. O. (2014). Prediction of resistance development against drug combinations by collateral responses to component drugs. *Science translational medicine, 6*(262), 262ra156-262ra156.

Naik, S., Mutyala, S., & Eswari, J. S. (2022). Impact of Antibiotic Resistance of Bacteria in Biofilms and Microbial Fuel Cell: Confronting the Dark Box for Global Health Threat. In *Antimicrobial Resistance* (pp. 137-152): Springer.

Nathan, C. (2004). Antibiotics at the crossroads. *Nature, 431*(7011), 899-902.

Obolski, U., Stein, G. Y., & Hadany, L. (2015). Antibiotic restriction might facilitate the emergence of multi-drug resistance. *PLoS computational biology, 11*(6), e1004340.

Pena-Miller, R., Laehnemann, D., Jansen, G., Fuentes-Hernandez, A., Rosenstiel, P., Schulenburg, H., & Beardmore, R. (2013). When the most potent combination of antibiotics selects for the greatest bacterial load: the smile-frown transition. *PLoS biology, 11*(4), e1001540.

Rodriguez de Evgrafov, M., Gumpert, H., Munck, C., Thomsen, T. T., & Sommer, M. O. (2015). Collateral resistance and sensitivity modulate evolution of high-level resistance to drug combination treatment in Staphylococcus aureus. *Molecular biology and evolution, 32*(5), 1175-1185.

Sanders, C. C. (2001). Mechanisms responsible for cross-resistance and dichotomous resistance among the quinolones. *Clinical infectious diseases, 32*(Supplement_1), S1-S8.

Spellberg, B., & Gilbert, D. N. (2014). The future of antibiotics and resistance: a tribute to a career of leadership by John Bartlett. *Clinical Infectious Diseases, 59*(suppl_2), S71-S75. doi:10.1093/cid/ciu392

Sprouffske, K., & Wagner, A. (2016). Growthcurver: an R package for obtaining interpretable metrics from microbial growth curves. *BMC bioinformatics, 17*(1), 1-4.

Tagliabue, A., & Rappuoli, R. (2018). Changing priorities in vaccinology: antibiotic resistance moving to the top. *Frontiers in immunology, 9*, 1068.

Tekin, E., Beppler, C., White, C., Mao, Z., Savage, V. M., & Yeh, P. J. (2016). Enhanced identification of synergistic and antagonistic emergent interactions among three or more drugs. *Journal of The Royal Society Interface, 13*(119), 20160332. doi:10.1098/rsif.2016.0332

Tekin, E., Savage, V. M., & Yeh, P. J. (2017). Measuring higher-order drug interactions: a review of recent approaches. *Current Opinion in Systems Biology, 4*, 16-23.

Tekin, E., White, C., Kang, T. M., Singh, N., Cruz-Loya, M., Damoiseaux, R., . . . Yeh, P. J. (2018). Prevalence and patterns of higher-order drug interactions in Escherichia coli. *NPJ systems biology and applications, 4*(1), 31.

Torella, J. P., Chait, R., & Kishony, R. (2010). Optimal drug synergy in antimicrobial treatments. *PLoS computational biology, 6*(6), e1000796.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and therapeutics, 40*(4), 277.

Wood, K., Nishida, S., Sontag, E. D., & Cluzel, P. (2012). Mechanism-independent method for predicting response to multidrug combinations in bacteria. *Proceedings of the National Academy of Sciences, 109*(30), 12254-12259.

Wood, K. B. (2016). Pairwise interactions and the battle against combinatorics in multidrug therapies. *Proceedings of the National Academy of Sciences, 113*(37), 10231-10233.

Wright, G. D. (2010). Antibiotic resistance in the environment: a link to the clinic? *Current opinion in microbiology, 13*(5), 589-594.

Yeh, P., Hegreness, M., Aiden, A. P., & Kishony, R. (2009). Drug interactions and the evolution of antibiotic resistance. *Nature Reviews Microbiology, 7*, 460. doi:10.1038/nrmicro2133

Yeh, P., Tschumi, A. I., & Kishony, R. (2006). Functional classification of drugs by properties of their pairwise interactions. *Nature genetics, 38*(4), 489. doi:10.1038/ng1755

Zimmer, A., Katzir, I., Dekel, E., Mayo, A. E., & Alon, U. (2016). Prediction of multidimensional drug dose responses based on measurements of drug pairs. *Proceedings of the National Academy of Sciences, 113*(37), 10442-10447.