Supplementary Information

Uncovering Emergent Interactions in Three-Way Combinations of Stressors

Journal of the Royal Society Interface

Casey Beppler*[1,2], Elif Tekin*[3], Zhiyuan Mao[1,2], Cynthia White[1], Cassandra McDiarmid[1], Emily Vargas[1], Jeffrey H. Miller[2], Van M. Savage[1,3,4], and Pamela J. Yeh**[1]

[1]Department of Ecology and Evolutionary Biology, University of California, Los Angeles, California 90095, USA

[2]Department of Microbiology, Immunology, and Molecular Genetics, the Molecular Biology Institute, and the David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA

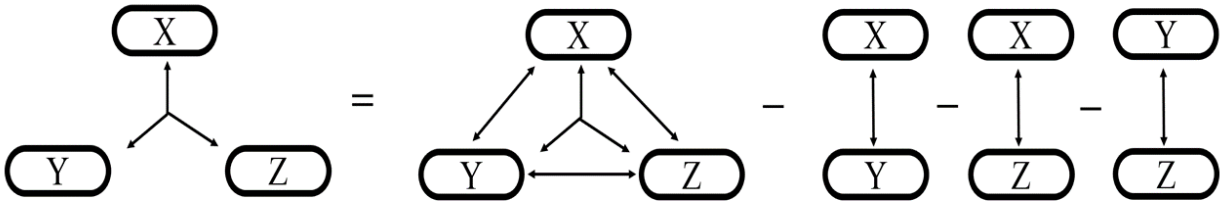
[3]Department of Biomathematics, University of California, David Geffen School of Medicine, Los Angeles, California 90095, USA

[4]Santa Fe Institute, Santa Fe, New Mexico 87501, USA

*Authors contributed equally.

**Corresponding Author. Phone: (310) 825-7441. Fax: (310) 206-0484. Email: pamelayeh@ucla.edu. Mailing Address: 612 Charles E. Young Drive East, Room 120, Box 957246, Los Angeles, California 90095-1606.

Supplementary Figure 1. Illustration of the emergent three-way (E3) interaction measure. The E3 measure, $\hat{\varepsilon}_{X,Y,Z}$, can be expressed as a difference between how drugs *X*, *Y*, and *Z* interact as compared to the effects originating solely from each pairwise interaction. As such, $\hat{\varepsilon}_{X,Y,Z}$ (the schematic on the far left) measures any emergent parts of the three-way interaction that cannot be explained in terms of pairwise interactions.



Supplementary Figure 2. Triple-drug interactions. A. Data for triple-antibiotic figures are shown for both deviation from additivity (DA) and emergent three-way (E3) interactions. Figures are presented as described in Figs 1 and 2. Drug abbreviations and concentrations are listed in Table 1. Note that the only potential difference in figures for DA and E3 is the coloring of the triple-drug interaction, and in cases where both measures agree, the coloring of the triple-drug interaction is the same. For synergy, both measures agree in the case of CLI+CPR+STR and CLI+CPR+ERY (see main text Fig. 3), and for antagonism, CPR+ERY+TOB. The following triple drug interactions differ between the two measures: ERY+FOX+STR, CPR+ERY+STR, CLI+FOX+TOB, CLI+ERY+STR, CLI+ERY+TOB, CPR+CLI+TOB, CPR+FOX+TOB, and CLI+CPR+FOX. **B.** The same data shown in traditional bar graph format.

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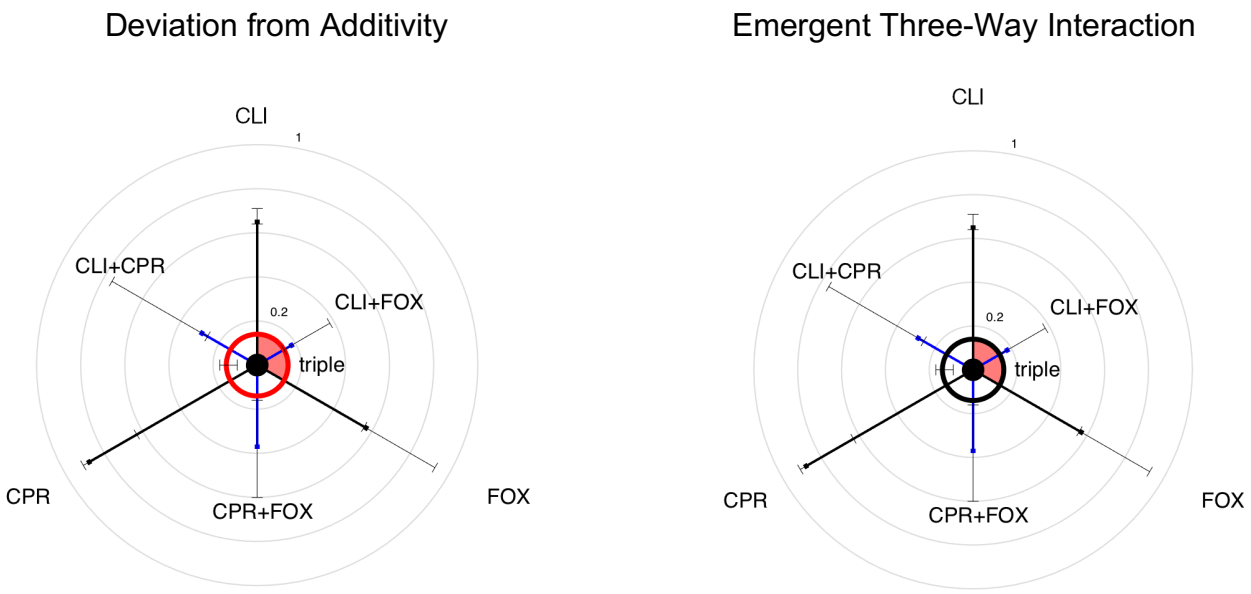

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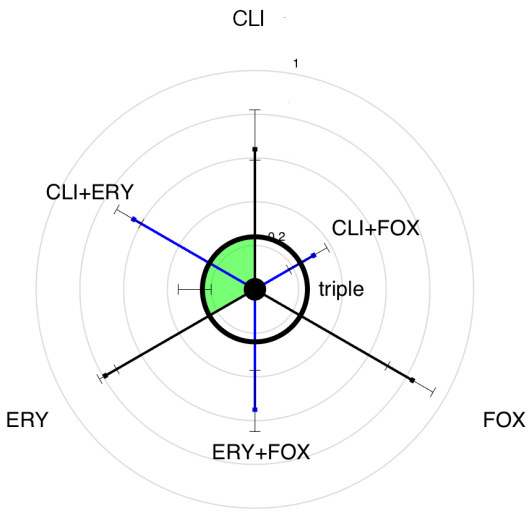

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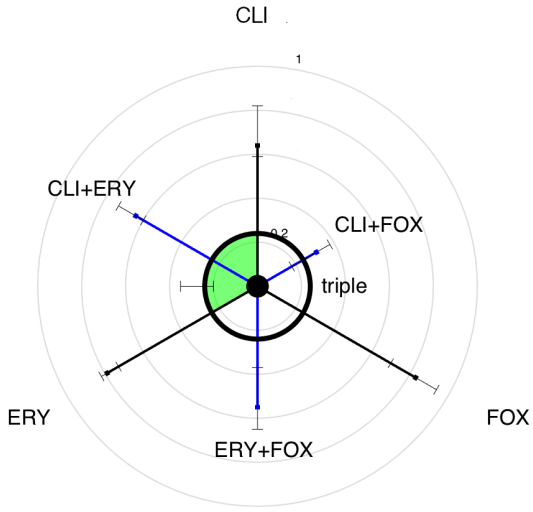

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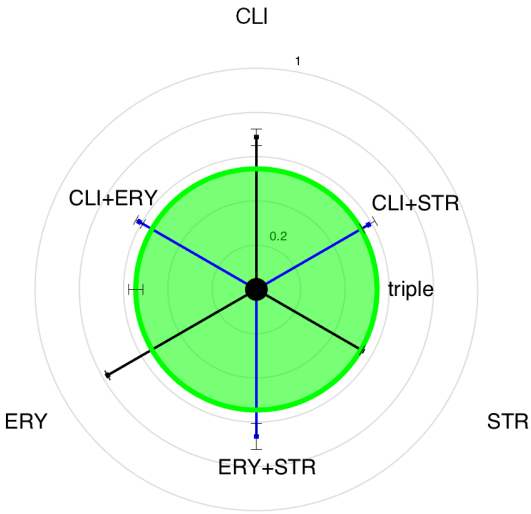

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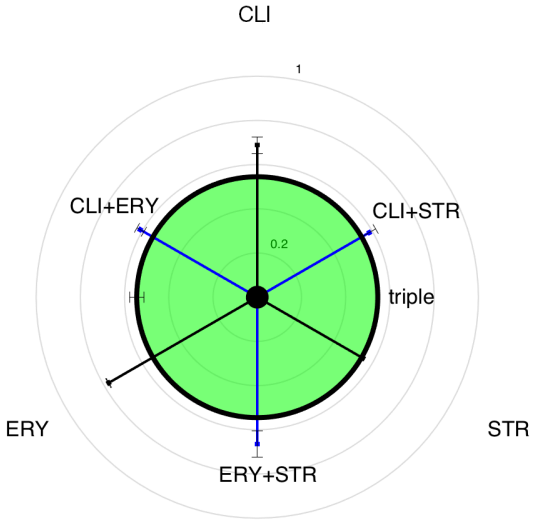

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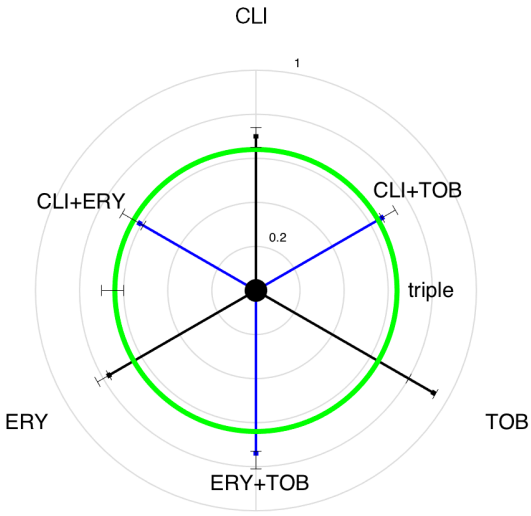

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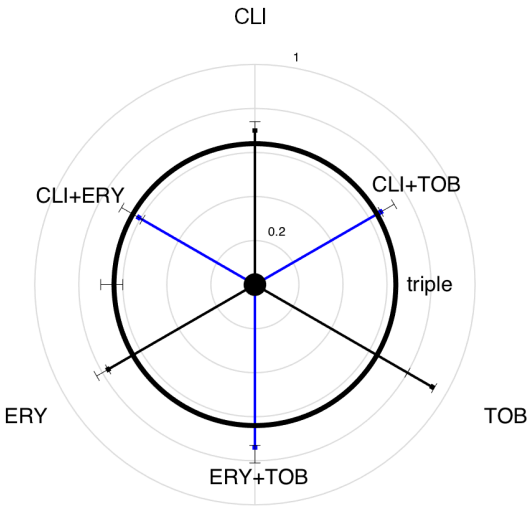

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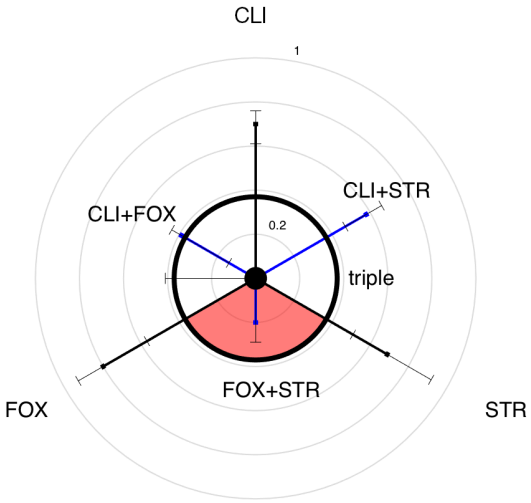

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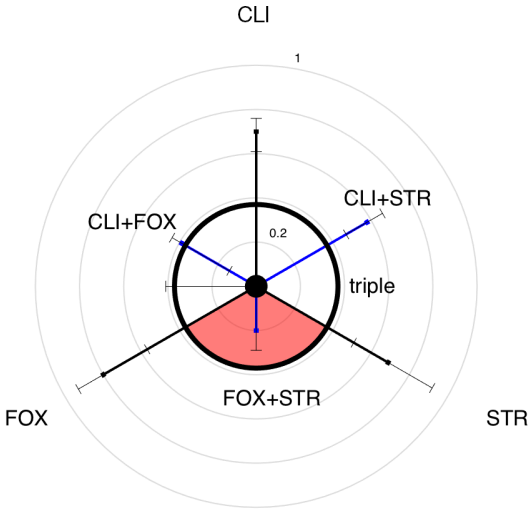

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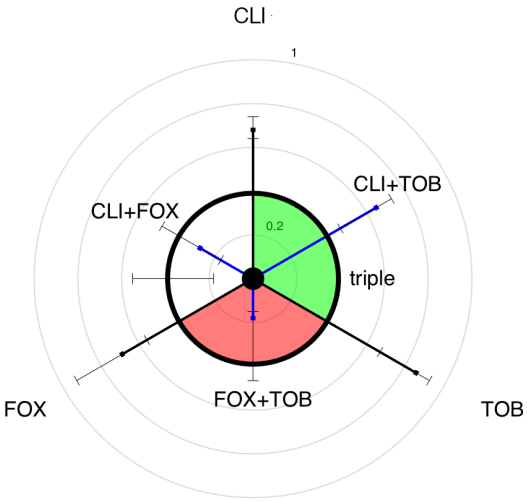

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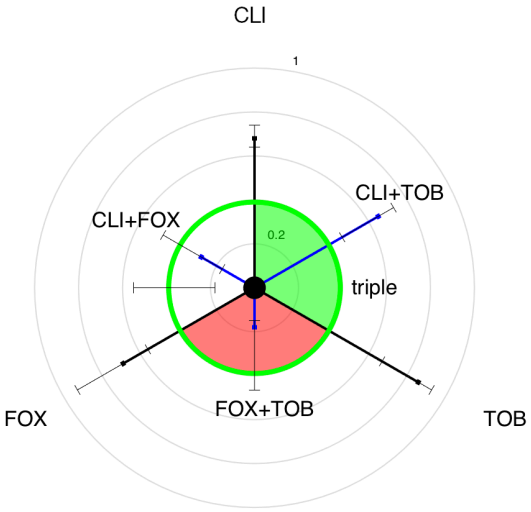

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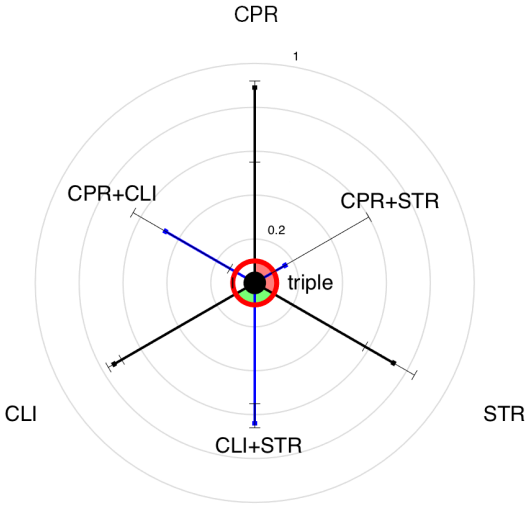

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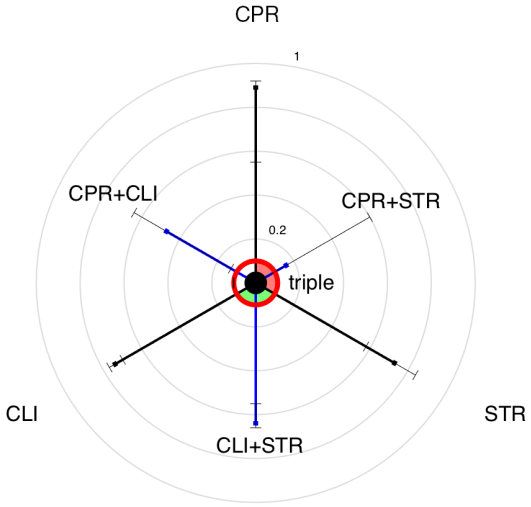

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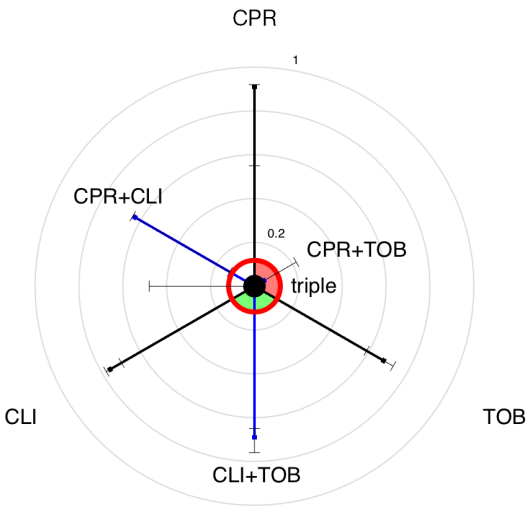

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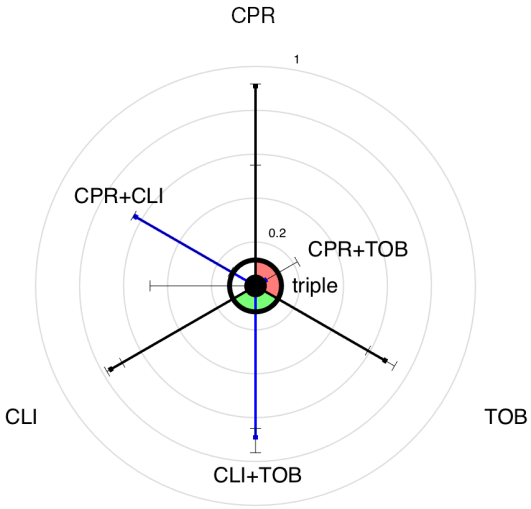

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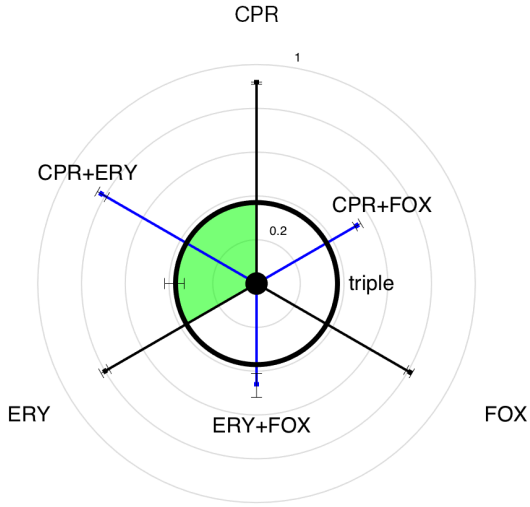

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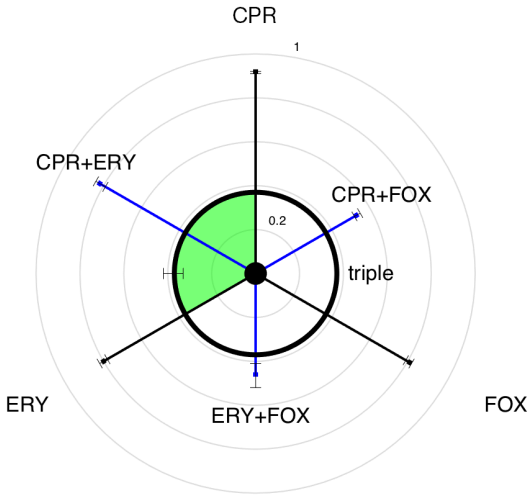

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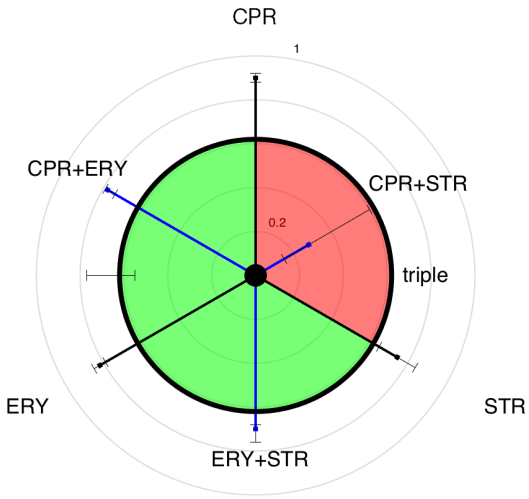

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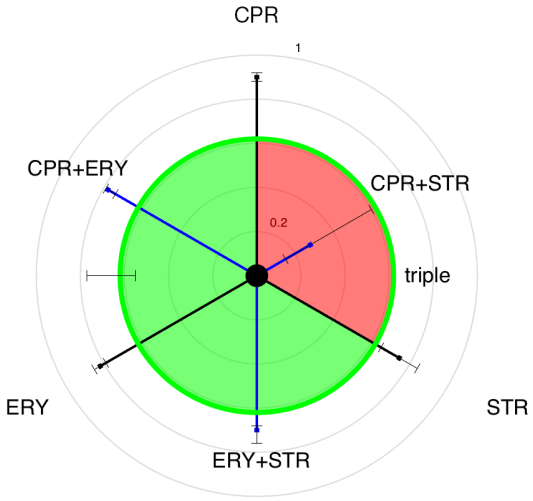

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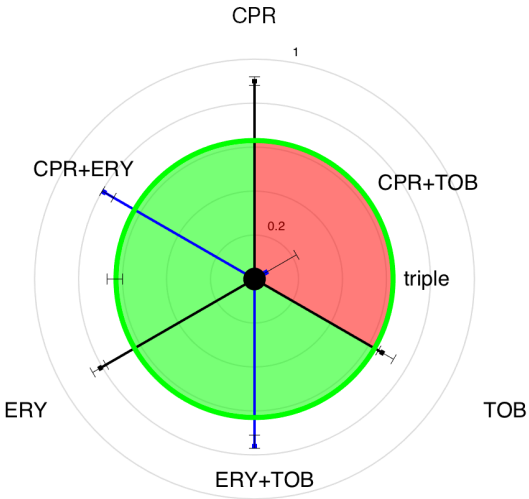

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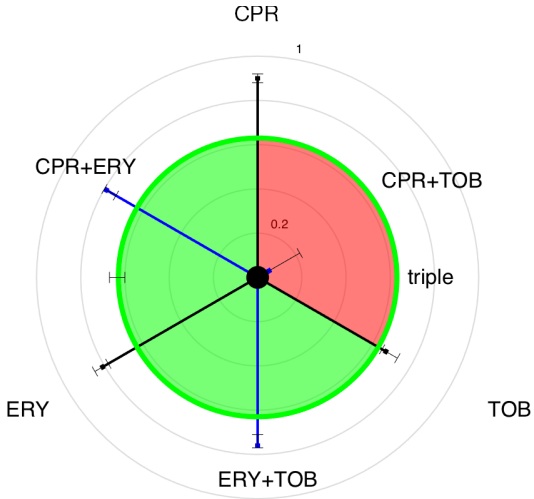

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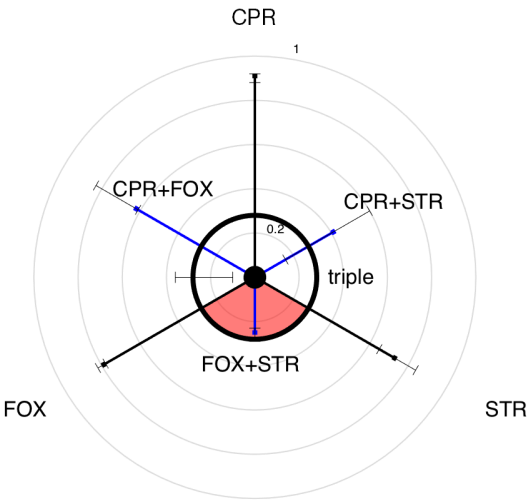

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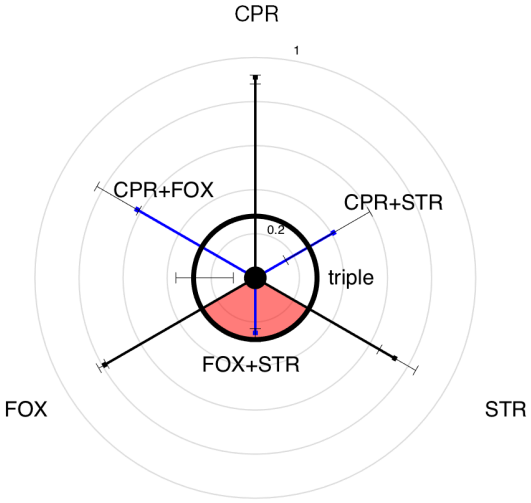

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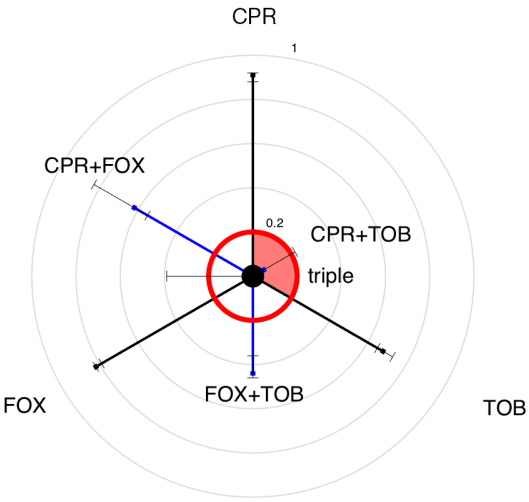

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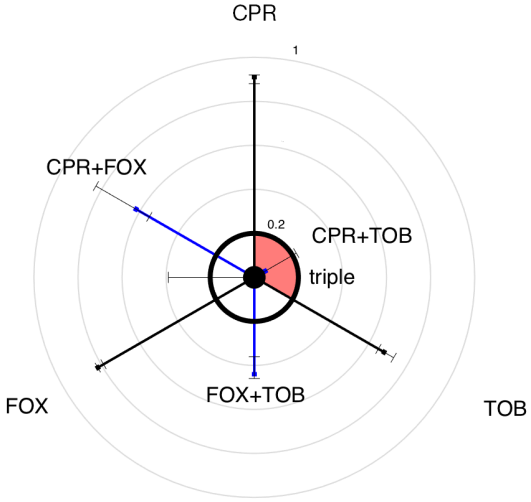

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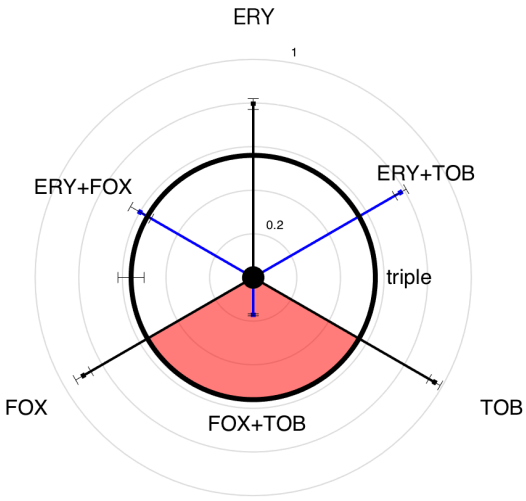

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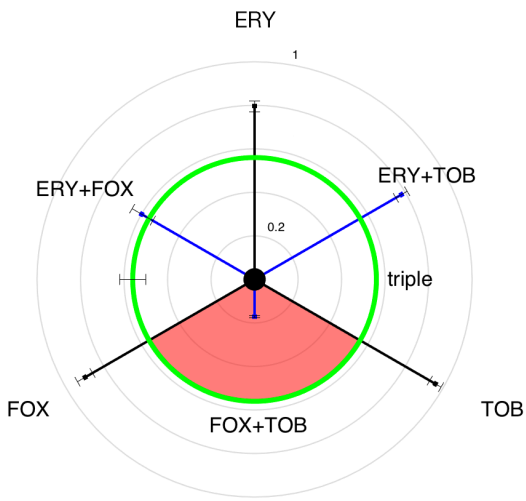

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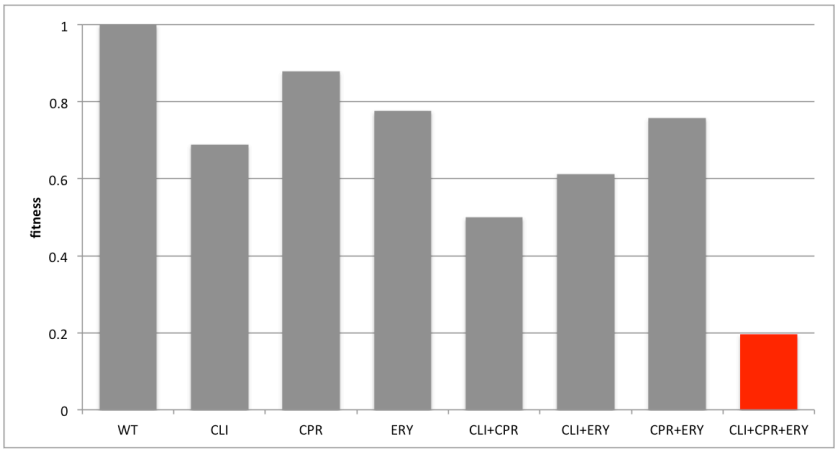
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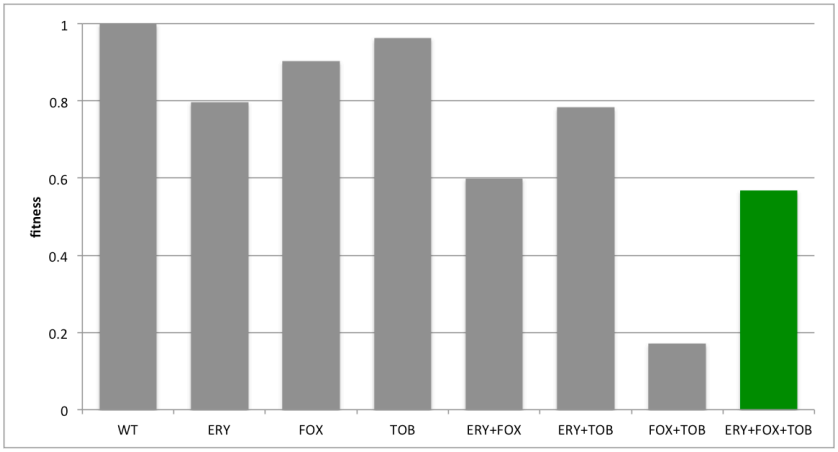
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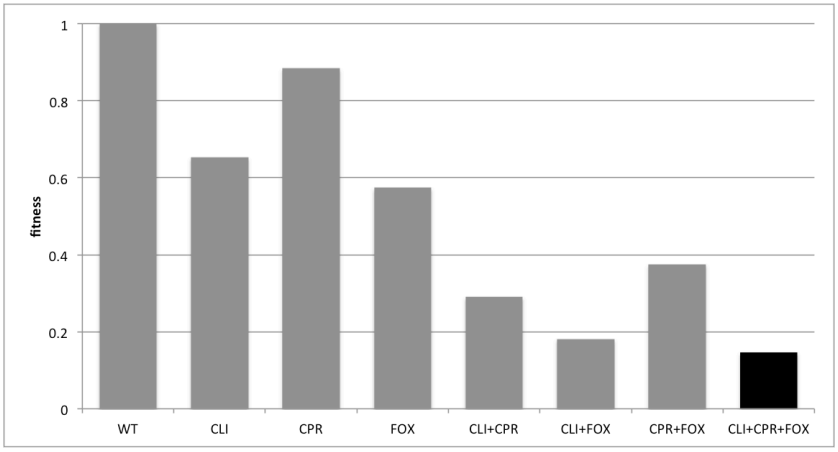
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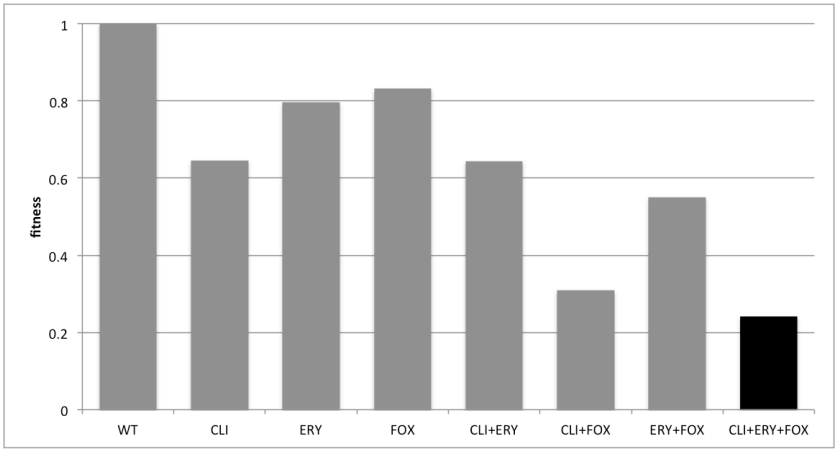
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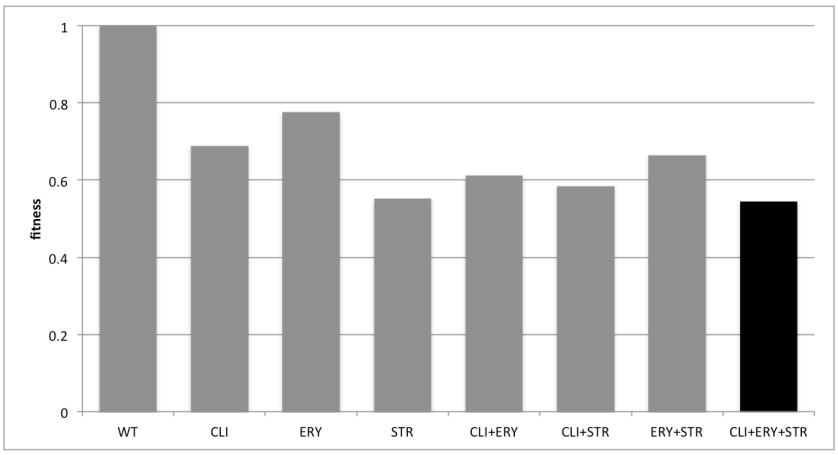
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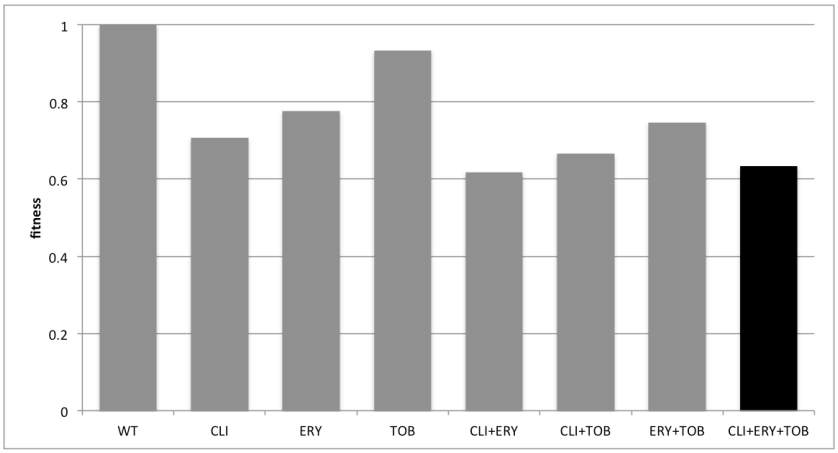
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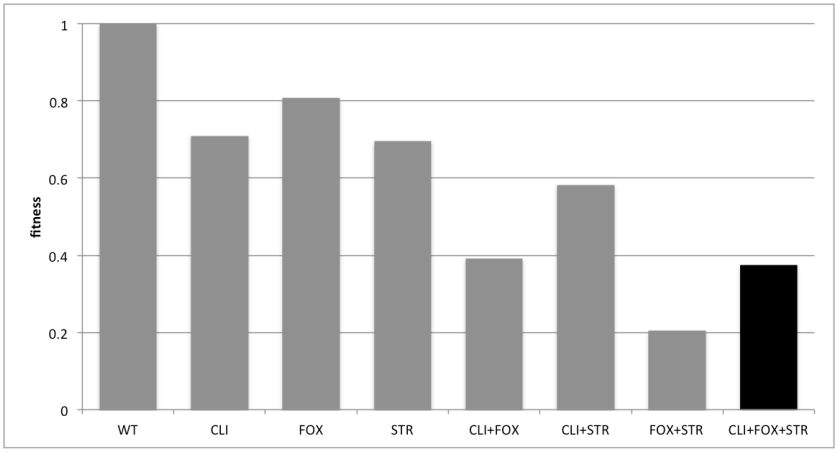
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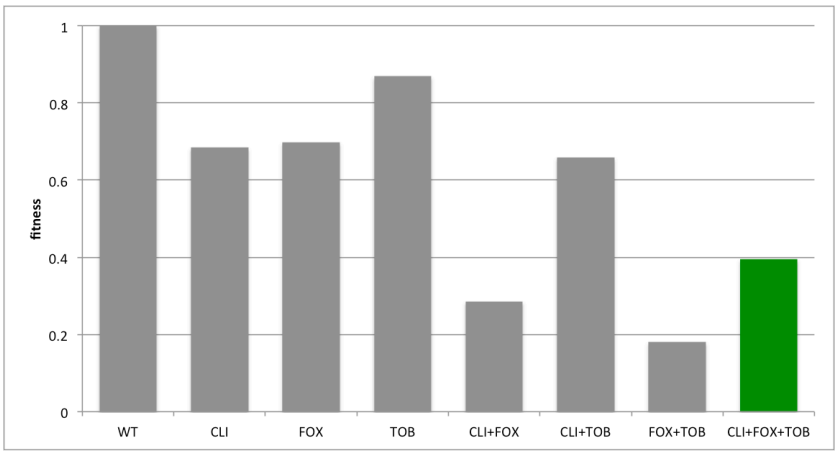
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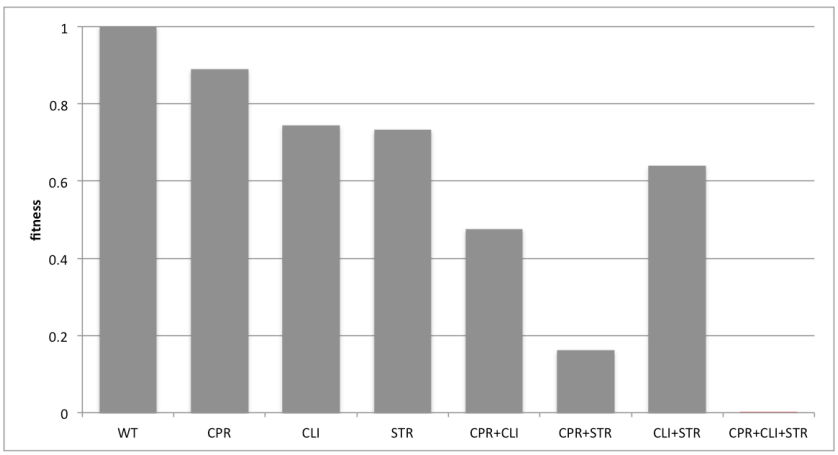
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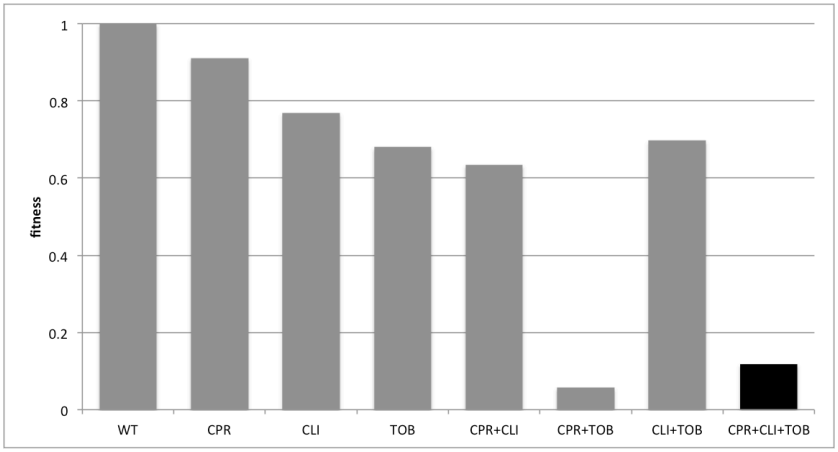
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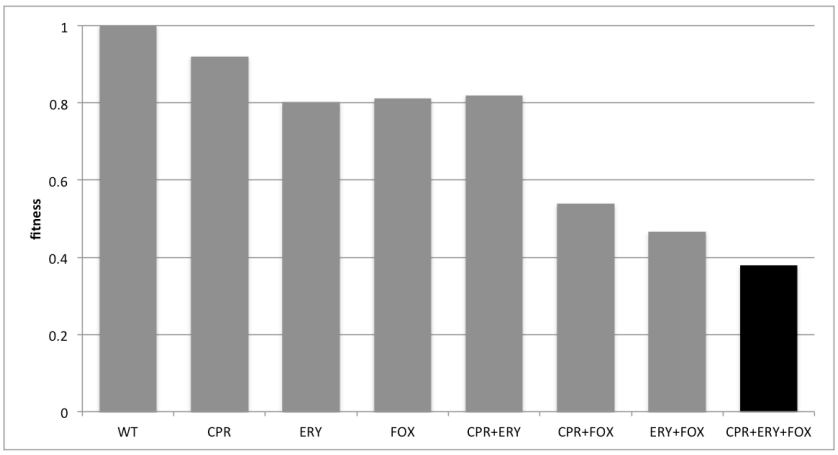
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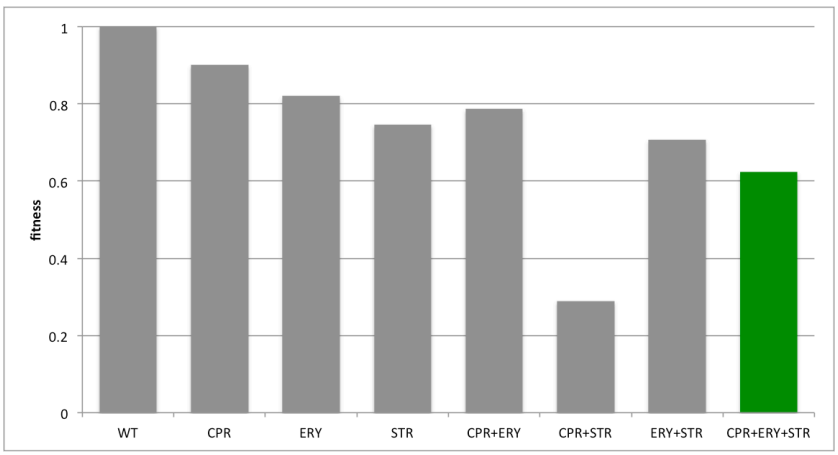
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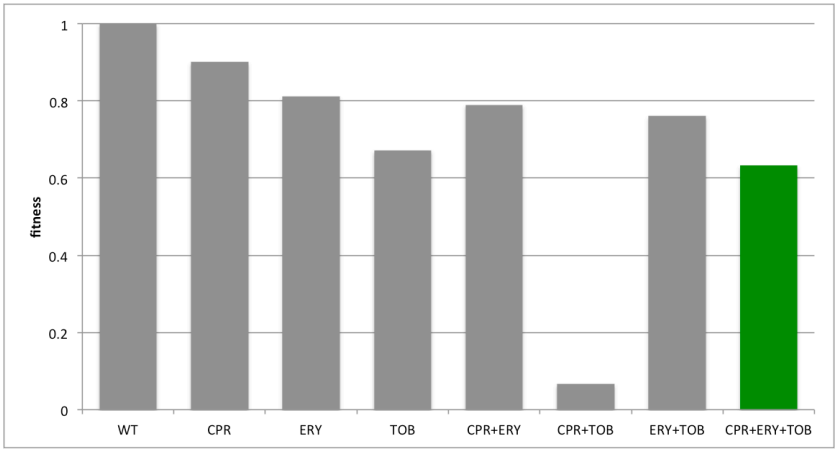
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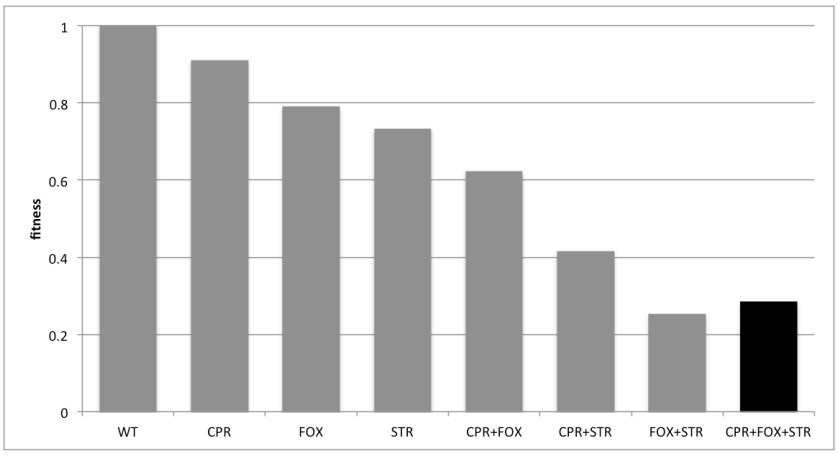
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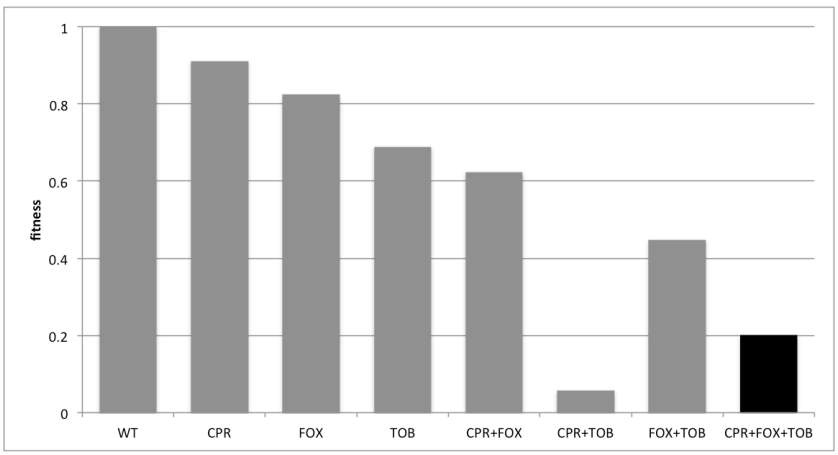
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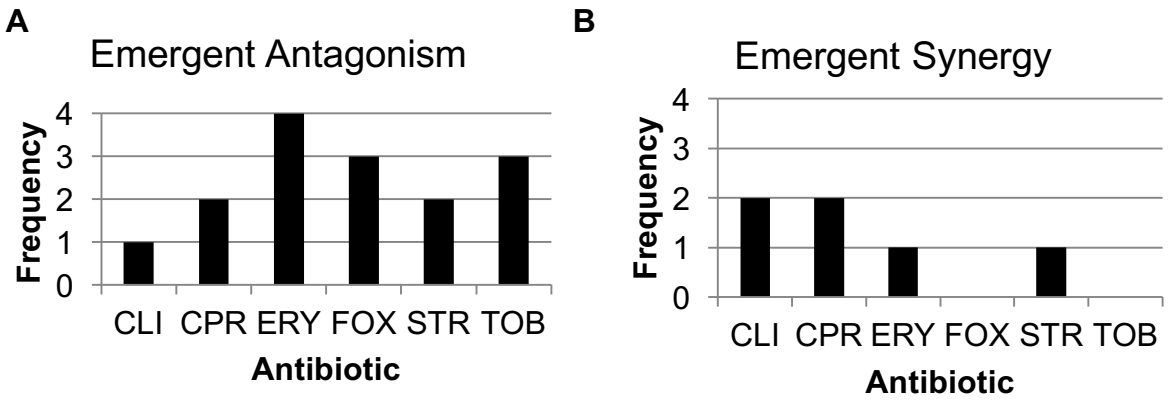
CPR+FOX+TOB

Supplementary Figure 3. Frequency of antibiotics involved in emergent synergistic and antagonistic three-drug interactions. A. Number of times each antibiotic appears in an emergent antagonism, according to emergent three-way interactions. **B.** Number of times each antibiotic appears in an emergent synergy, according to emergent three-way interactions. Antibiotic abbreviations are given in Table 1.



Supplementary Table 1. Data for triple-drug interaction assays. Growth percentages as compared to the no-drug control (100% growth) are given as median and maximum/minimum for the three single-drug conditions, the three two-drug combinations, and the triple combination. All triple combination experiments were repeated independently 2 or 3 times, each with 4-6 wells for each condition. (Note: only data for one concentration of each antibiotic is shown. Thus, although each three-drug combination was tested a minimum of three times, n may be 2 for a certain concentration.) Measure of deviation from additivity and emergent three-way interactions, described in the methods, are given for each triple combination. For cases in which a two-drug combination results in lethality, the emergent three-way interaction measure is non-applicable because it is not possible to determine the effect of the third drug. Antibiotic abbreviations are given in Table 1. All antibiotic concentrations are given in μg/ml.

	Single 1 Growth %	Single 2 Growth %	Single 3 Growth %	Double 1 Growth %	Double 2 Growth %	Double 3 Growth %	Triple Growth %	Rescaled Deviation From Additivity	Rescaled Emergent Three-Way Interaction
CLI 31.5+CPR 0.013+ERY 14	CLI	CPR	ERY	CLI+CPR	CLI+ERY	CPR+ERY	CLI+CPR+ ERY		
Median (n=2)	68.9	87.9	77.6	50.0	61.2	75.8	19.6	-0.5826	-0.6139
Max	72.5	88.4	77.8	76.3	62.8	76.3	27.0		
Min	65.3	87.3	77.5	23.6	59.6	75.4	12.2		
CLI 31.5+CPR 0.013+FOX 1.16	CLI	CPR	FOX	CLI+CPR	CLI+FOX	CPR+FOX	CLI+CPR+ FOX		
Median (n=3)	65.3	88.4	57.4	29.0	18.2	37.6	14.6	-0.5585	0.3561
Max	71.5	91.8	93.6	76.3	38.9	60.8	17.3		
Min	64.3	63.8	56.3	26.0	14.1	16.3	9.7		
CPR 0.01+CLI 31.5+STR 4.5	CPR	CLI	STR	CPR+CLI	CPR+STR	CLI+STR	CPR+CLI+ STR		
Median (n=5)	89.0	74.4	73.3	47.5	16.1	64.0	0.1	-0.9971	-0.9791
Max	92.1	77.8	84.2	64.6	60.2	66.5	0.3		

Min	55.1	70.6	58.8	13.5	0.3	55.5	0.0		

CPR 0.01+CLI 31.5+TOB 1.3	CPR	CLI	TOB	CPR+CLI	CPR+TOB	CLI+TOB	CPR+CLI+ TOB		
Median (n=5)	91.1	76.9	68.0	63.4	5.7	69.7	11.8	-0.7524	-0.2499
Max	92.4	78.9	73.5	64.6	22.9	76.6	48.4		
Min	55.1	70.6	59.7	13.5	0.3	65.7	0.1		

CLI 31.5+ERY 14+FOX 1.16	CLI	ERY	FOX	CLI+ERY	CLI+FOX	ERY+FOX	CLI+ERY+ FOX		
Median (n=4)	64.5	79.6	83.1	64.3	31.0	55.0	24.2	-0.4343	-0.1435
Max	82.5	82.0	94.6	73.5	38.3	65.1	35.3		
Min	59.0	73.3	70.9	60.7	18.9	37.0	20.4		

CLI 31.5+ERY 14+STR 4.5	CLI	ERY	STR	CLI+ERY	CLI+STR	ERY+STR	CLI+ERY+ STR		
Median (n=2)	68.9	77.6	55.2	61.2	58.5	66.5	54.5	0.9730	-0.1742
Max	72.5	77.8	55.2	62.8	61.8	72.4	57.7		
Min	65.3	77.5	55.1	59.6	55.1	60.5	51.2		

CLI 31.5+ERY 14+TOB 1.3	CLI	ERY	TOB	CLI+ERY	CLI+TOB	ERY+TOB	CLI+ERY+ TOB		
Median (n=4)	70.6	77.6	93.3	61.8	66.5	74.6	63.4	0.6261	0.3349
Max	74.5	83.1	94.5	70.5	73.8	81.7	70.1		
Min	65.3	77.3	80.9	59.6	66.1	73.6	60.0		

CLI 31.5+FOX 1.16+STR 4.5	CLI	FOX	STR	CLI+FOX	CLI+STR	FOX+STR	CLI+FOX+ STR		
Median (n=6)	70.9	80.7	69.6	39.2	58.2	20.4	37.5	-0.0594	0.4696
Max	76.9	93.6	92.8	44.5	66.5	29.0	41.3		
Min	61.5	57.4	51.4	14.1	47.6	0.7	0.9		

CLI 31.5+FOX 1.16+TOB 1.3	CLI	FOX	TOB	CLI+FOX	CLI+TOB	FOX+TOB	CLI+FOX+ TOB		
Median (n=4)	68.4	69.8	86.9	28.6	65.9	18.1	39.5	-0.0479	0.5766
Max	74.5	93.6	93.7	48.7	73.8	46.9	55.6		
Min	64.3	56.3	67.3	14.1	46.0	14.0	18.7		

CLI 31.5+STR 4.5+TOB 1.3	CLI	STR	TOB	CLI+STR	CLI+TOB	STR+TOB	CLI+STR+ TOB		
Median (n=7)	73.1	75.2	68.9	61.3	72.1	0.7	0.8	-0.9784	N/A
Max	76.9	92.8	93.7	66.5	75.0	2.5	13.0		
Min	61.5	39.9	65.0	48.9	61.5	0.0	0.1		

CPR 0.01+ERY 14+FOX 1.16	CPR	ERY	FOX	CPR+ERY	CPR+FOX	ERY+FOX	CPR+ERY +FOX		
Median (n=2)	92.0	80.3	81.1	82.0	53.9	46.7	37.9	-0.3673	0.1068
Max	92.4	82.2	82.6	84.3	55.7	52.2	42.2		
Min	91.7	78.5	79.6	79.6	52.1	41.2	33.7		

CPR 0.01+ERY 14+STR 4.5	CPR	ERY	STR	CPR+ERY	CPR+STR	ERY+STR	CPR+ERY +STR		
Median(n=4)	90.0	82.0	74.6	78.7	28.9	70.7	62.4	0.3735	0.6843
Max	92.1	85.5	84.2	79.9	60.2	76.9	77.8		
Min	88.0	79.6	65.9	74.5	15.3	68.7	55.5		

CPR 0.01+ERY 14+TOB 1.3	CPR	ERY	TOB	CPR+ERY	CPR+TOB	ERY+TOB	CPR+ERY +TOB		
Median (n=4)	90.0	81.1	67.2	79.0	6.6	76.1	63.3	0.7857	0.8987
Max	92.4	85.5	73.5	79.9	22.9	77.5	67.6		
Min	88.0	78.5	65.0	74.5	0.3	71.8	60.2		

CPR 0.01+FOX 1.16+STR 4.5	CPR	FOX	STR	CPR+FOX	CPR+STR	FOX+STR	CPR+FOX +STR		
Median (n=3)	91.1	79.0	73.3	62.3	41.7	25.3	28.6	-0.4586	0.4200
Max	92.1	82.4	84.2	83.2	60.2	29.0	36.9		
Min	88.0	78.9	65.9	61.6	16.1	23.2	10.8		

CPR 0.01+FOX 1.16+TOB 1.3	CPR	FOX	TOB	CPR+FOX	CPR+TOB	FOX+TOB	CPR+FOX +TOB		
Median (n=3)	91.1	82.4	68.9	62.3	5.7	44.8	20.1	-0.6108	0.4187
Max	92.4	82.6	73.5	83.2	22.9	46.8	39.7		
Min	88.0	79.0	65.0	55.7	0.3	36.6	0.4		

CPR 0.01+STR 4.5+TOB 1.3	CPR	STR	TOB	CPR+STR	CPR+TOB	STR+TOB	CPR+STR +TOB		
Median (n=3)	89.0	73.3	65.5	16.1	7.5	0.0	0.0	-0.9994	N/A
Max	91.1	75.9	68.9	41.7	22.9	2.5	0.0		
Min	88.0	65.9	65.0	15.3	5.7	0.0	0.0		

ERY 14+FOX 1.16+STR 4.5	ERY	FOX	STR	ERY+FOX	ERY+STR	FOX+STR	ERY+FOX +STR		
Median (n=4)	80.6	80.7	69.6	52.8	70.1	24.3	47.9	0.1101	0.5416
Max	85.5	93.6	84.2	54.4	76.9	29.0	51.4		
Min	77.5	78.9	55.1	33.7	60.5	7.9	41.1		

ERY 14+FOX 1.16+TOB 1.3	ERY	FOX	TOB	ERY+FOX	ERY+TOB	FOX+TOB	ERY+FOX +TOB		
Median (n=2)	79.7	90.3	96.3	60.0	78.3	17.1	56.8	-0.1802	0.6997
Max	82.0	94.6	99.6	65.1	81.4	17.6	62.8		
Min	77.3	86.0	93.0	54.8	75.2	16.7	50.8		
ERY 14+STR 4.5+TOB 1.3	ERY	STR	TOB	ERY+STR	ERY+TOB	STR+TOB	ERY+STR +TOB		
Median (n=9)	82.3	75.2	68.9	71.5	75.6	0.8	1.5	-0.9654	N/A
Max	87.9	92.8	93.7	76.9	81.7	78.9	65.4		
Min	75.6	39.9	59.7	33.2	69.9	0.0	0.3		
FOX 1.16+STR 4.5+TOB 1.3	FOX	STR	TOB	FOX+STR	FOX+TOB	STR+TOB	FOX+STR +TOB		
Median (n=6)	80.3	74.3	85.1	12.8	39.4	0.7	0.3	-0.9939	N/A
Max	93.6	92.8	93.7	29.0	57.1	50.8	11.4		
Min	57.4	51.4	65.0	0.4	14.0	0.0	0.0		

Supplementary Table 2. Dose-Dependence data for single drugs ([X], [2X], [3X]). Antibiotic abbreviations are listed in Table 1, TMP=trimethoprim, CHL=chloramphenicol, AMP=ampicillin, and GEN=gentamycin. Note that because of variable inhibitory effects of single-drug treatments between experiments, single drug doses may not be identical to the triple-drug experiment doses.

	Single [X] Growth %	Double [2X] Growth %	Triple [3X] Growth %	Pairwise Deviation From Additivity Result	Triple Deviation From Additivity Result	Emergent Three-Way Interaction Result
TMP 0.08	TMP	TMP+TMP	TMP+TMP+TMP			
Median (n=3)	89.34	35.58	0.70	Synergy	Synergy	Additive
Max	92.87	39.43	1.14			
Min	83.65	25.46	0.69			
CPR 0.01	CPR	CPR+CPR	CPR+CPR+CPR			
Median (n=3)	95.61	64.20	12.48	Additive	Synergy	Additive
Max	96.07	65.10	22.24			
Min	93.88	61.84	11.06			

CHL 40	CHL	CHL+CHL	CHL+CHL+CHL			
Median (n=3)	73.60	49.60	37.89	Additive	Additive	Additive
Max	74.93	60.50	46.32			
Min	72.54	48.78	36.54			
CLI 20	CLI	CLI+CLI	CLI+CLI+CLI			
Median (n=3)	72.91	48.04	39.19	Additive	Additive	Additive
Max	80.45	49.66	39.55			
Min	71.15	46.58	38.35			
AMP 1.0	AMP	AMP+AMP	AMP+AMP+AMP			
Median (n=3)	77.83	36.87	0.71	Additive	Additive	Additive
Max	79.06	46.49	1.09			
Min	76.77	36.19	0.65			
TOB 0.5	TOB	TOB+TOB	TOB+TOB+TOB			
Median (n=3)	69.04	58.60	62.25	Synergy	Antagonism	Additive
Max	73.62	67.20	63.13			
Min	68.89	57.37	43.21			
ERY 30	ERY	ERY+ERY	ERY+ERY+ERY			
Median (n=3)	59.09	44.27	28.98	Additive	Additive	Additive
Max	63.81	44.41	29.32			
Min	56.73	42.13	28.05			
FOX 1	FOX	FOX+FOX	FOX+FOX+FOX			
Median (n=3)	62.63	32.29	17.04	Additive	Additive	Additive
Max	63.48	33.23	18.51			
Min	56.36	30.04	15.57			
STR 2	STR	STR+STR	STR+STR+STR			
Median (n=3)	70.15	60.53	36.58	Antagonism	Additive	Additive
Max	70.70	69.81	37.84			
Min	65.41	56.94	31.00			
GEN 0.4	GEN	GEN+GEN	GEN+GEN+GEN			
Median (n=3)	71.92	63.10	30.37	Synergy	Additive	Synergy
Max	74.55	65.42	31.31			
Min	67.17	53.33	27.36			

Supplementary Table 3. Comparison of 3-Way Interactions to 3-Way ANOVA analysis. The 3-way ANOVA result in both linear and logarithmic space is compared with results for our DA and E3 interaction measures. We quantify agreement by using a "success" rate, which is defined according to whether 3-way ANOVA agrees with our DA or E3 measure. Since ANOVA only detects the presence of interactions but does not distinguish synergy from antagonism, we compare only whether ANOVA and DA or E3 both classify a 3-way interaction as not significant (additive) or an interaction as significant (i.e., a non-additive interaction (either synergy or antagonism)). From this perspective, the success rate represents the percent of 3-Way ANOVAs that are identified as successful for predicting **A.** emergent 3-way (E3) interaction or **B.** deviation from additivity.

A

Emergent Three-Way Interaction

	3-Way ANOVA Success Rate	log 3-Way ANOVA Success Rate
Additive	67% (6/9)	89% (8/9)
Synergy	0% (0/2)	0% (0/2)
Antagonism	100% (5/5)	100% (5/5)

B

Deviation From Additivity

	3-Way ANOVA Success Rate	log 3-Way ANOVA Success Rate
Additive	38% (3/8)	50% (4/8)
Synergy	33% (3/9)	0% (0/9)
Antagonism	67% (2/3)	67% (2/3)

Supplementary Text 1. Emergent four-way interaction.

As an example of our framework applied to more than three components, we derive the emergent four-way interaction measure to be

$$\hat{\varepsilon}_{V,X,Y,Z} = w_{VXYZ} - w_V w_{XYZ} - w_X w_{VYZ} - w_Y w_{VXZ} - w_Z w_{VXY} + w_V w_X w_{YZ} + w_V w_Y w_{XZ} + w_V w_Z w_{XY} + w_X w_Y w_{VZ} + w_X w_Z w_{VY} + w_Y w_Z w_{VX} - 3 w_V w_X w_Y w_Z$$

Notably, this measure for four-way emergent interactions (and indeed all of our measures for emergent interactions among more than three components) differs substantially from the algebraic formulas in Wood et al. (1), which only depend on pairwise fitnesses but not on fitnesses in the presence of three or more drugs. Data for four-way interactions would provide a clear empirical test to distinguish between our expectations and those from the phenomenological formulas from Wood et al. (1).

Supplementary Text 2. Three-way ANOVA.

As an additional point of comparison for the emergent three-way (E3) interaction measure, the use of ANOVA within multiple predator effects (MPE) studies suggests application of a three-way ANOVA to identify emergent interactions within our data. Because our fitness functions are exponential, an ANOVA is best performed in logarithmic space. However, with only three replicates of each experiment, variances are not reliably estimated and it is not possible to test if the data are parametric, so the conditions for testing significance in ANOVA may not be satisfied. Furthermore, any assumed increase in effective replication due to the constancy of the variance across two- and three-drug combinations is likely to be strongly violated when the drugs interact. This is partly because small changes or errors in drug concentration lead to highly non-linear effects on growth rate, such that synergistic interactions can substantially increase the variance and antagonistic interactions can substantially decrease the variance compared with the additive case. In addition, translating logarithmic three-way ANOVA into an underlying interaction measure yields logarithms of similar types of terms (e.g., $\log(w_X w_{YZ})$ and $\log(w_X w_Y w_Z)$) as our E3 metric but with different coefficients. Thus, based on our starting definition of no interaction (i.e., Bliss independence), standard three-way ANOVA is not equivalent to the correct measure of emergent interactions. In Supplementary Table 3, we show a comparison between our interaction measures and 3-way ANOVA.

References

1. Wood K, Nishida S, Sontag ED, Cluzel P. Mechanism-independent method for predicting response to multidrug combinations in bacteria. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(30):12254-9.