Positioning of negative feedback loops within immune signaling pathways influences host fitness through noise in AMP expression

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Abstract

Signaling pathways depend on negative feedback loops (NFLs) to regulate internal noise. Across diverse organisms, signaling pathways are regulated by NFLs that function at different cellular locations. These range from NFLs functioning upstream near signal-receiving receptors to those downstream within the nucleus. Multi-level regulation of signaling pathways by NFLs is ubiquitous; however, we do not know how it influences noise regulation and ultimately host fitness. Here, we quantify noise in the expression of antimicrobial peptides (AMPs) upon induction of immune signaling using stochastic models. We hypothesize that noise regulation in the expression of immune genes is crucial for mounting nuanced responses to diverse environmental challenges. By altering the strength of NFLs that function at different cellular locations, we measured the effect of noise on fitness across various environmental conditions. We discovered that upstream NFLs reduce noise whereas downstream NFLs increase noise in the expression of AMPs. The noisy expression of AMPs by downstream NFLs increases host fitness during repeated exposure to pathogens. Conversely, upstream NFLs reduce fitness variation across genotypes possibly giving rise to bet-hedging. This study shows the significance of multi-level regulation by NFLs and contributes to our understanding of noise regulation in diverse signaling pathways.

Introduction

Organisms rely on signaling pathways to monitor environmental changes and generate appropriate responses (De Meyts, 2016; Kawasaki & Kawai, 2014; Komiya & Habas, 2008; Saxton & Sabatini, 2017). Noise or stochasticity (inherent unpredictability) is a fundamental characteristic of signaling pathways (Tsimring, 2014), which originates from random fluctuations in the small number of signaling proteins and interactions between them (Ladbury & Arold, 2012). The benefit of a noisy response to stimuli can be explained via several mechanisms. For example, under unpredictable environments, bet-hedging (reduction of fitness variance in favor of geometric fitness) generates noisy phenotypes among offspring to spread risk among individuals (Olofsson et al., 2009). On the other hand, if the relationship between phenotype and

fitness is non-linear, Jensen's inequality predicts that the average fitness of the population is better improved by increasing phenotypic variance (noise) than increasing phenotypic mean due to non-linear averaging (Ruel & Ayres, 1999). Finally, the theory of stochastic resonance predicts that in suboptimal non-linear systems, noise evolves because it improves signal detection (Fig. S1) (Hänggi, 2002). Excessive noise, on the other hand, has been shown to adversely impact the processing of information (Wang & Zhang, 2011). Hence, signaling pathways maintain tight regulation over noise.

Signaling pathways regulate noise through several mechanisms. One such mechanism involves feedback loops (Hooshangi & Weiss, 2006; Zhang et al., 2012). Feedback loops activate proteins that either amplify the signal (positive feedback) or attenuate it (negative feedback loops, or NFLs). NFLs generally reduce noise (Becskei & Serrano, 2000). In addition, NFLs have been shown to increase noise frequency, which facilitates the removal of noise by downstream gene circuits (perhaps due to the averaging out effect caused by rapid oscillations) (Austin et al., 2006; Simpson et al., 2003). Thus, NFLs that function upstream of the pathway are expected to influence noise in the expression of target genes differently than those functioning downstream and in proximity to target genes. However, it remains an outstanding question how multi-level regulation of NFLs influences noise and, in turn, host fitness in the face of diverse environmental challenges.

In immune signaling pathways, NFLs regulate responses to minimize energetic costs and prevent immunopathology (Lochmiller & Deerenberg, 2000). Production of immune proteins incurs energetic costs, which can negatively affect growth and reproduction (DiAngelo et al., 2009; Urlacher et al., 2018). The accumulation of effector proteins, which are active against pathogens and parasites, may adversely affect host tissues, leading to immunopathology (Gabrysová et al., 2009; Read et al., 2008). NFLs also regulate noise within immune signaling pathways. In mice, for example, activation of NF-κB by tumor necrosis factor (TNF), induces A20, which acts as an NFL to reduce noise in the pathway and improve information at the early stages of signaling (Cheong et al., 2011). Regulation of noise in immune signaling pathways also influences the expression of target genes. For example, noise propagation within signaling pathways is proposed to cause bimodal expression of immune genes across identical bone marrow-derived dendritic cells (Shalek et al., 2013; Zhao et al., 2012). This variability in the response could be beneficial if cell populations are exposed to fluctuating environments or heterogeneous populations of pathogens.

Immune signaling pathways across animals and plants are regulated by NFLs functioning at different steps of signaling (Table 1). In all such pathways, signaling involves the activation of a central transcription factor that induces target genes against pathogens, as well as genes encoding NFLs. Although there are biological differences across immune signaling pathways, NFLs governing them generally fall into two categories: those that decrease input into the pathway by acting upstream and those that function downstream to reduce the output, while maintaining the signaling process. Simple theoretical models that consider such shared physical attributes offer valuable insights into their regulatory mechanisms. These models can be used to

elucidate the relationship between noise and the positioning of NFLs within pathways, which enables a more precise assessment of the effect of noise on response to stimuli. Such knowledge is crucial in determining evolutionary pressures acting on NFLs within signaling pathways.

Here we analyzed simple models that incorporate these common features in the context of Imd and Toll signaling, which are the two major immune signaling pathways in insects (De Gregorio et al., 2002). Immune signaling in *Drosophila melanogaster* is regulated by NFLs that function at different steps of signaling. For example, upon infection, the induction of Pirk instigates the removal of immune receptors from the cell surface (Lhocine et al., 2008). This dampens immunity by preventing ligand formation between receptors and the bacterial peptidoglycan, thus reducing input. The downstream NFLs, on the other hand, include a protein complex known as the repressosome (Kim et al., 2007) within the Imd pathway and the Cactus protein within the Toll pathway (Belvin & Anderson, 1996; Cai et al., 2022). These NFLs inhibit the activity of NF-κB transcription factors and reduce the output while maintaining signaling (Fig. 1). Differences in the cellular location of NFLs (e.g., Pirk vs repressosome) and mechanistic differences in the function of NFLs operating at the same location (cactus vs repressosome) could potentially yield distinct effects on noise regulation and fitness. In both Imd and Toll pathways, NF-κB transcription factors are responsible for the induction of antimicrobial peptides (AMPs) against pathogens (Stączek et al., 2023). Our goal is to identify environmental conditions that favor noise in the expression of immune genes. To this end, we investigate the impact of upstream and downstream NFLs within signaling pathways on noise regulation and its influence on fitness across diverse environmental conditions. By testing models of immune response across diverse conditions, we attempt to provide ecologically relevant insights into the dynamics of immune responses.

Table 1. NFLs* at different steps of signaling in immune-related pathways. Toll pathway in insects is only regulated by an NFL downstream of the pathway.

Signaling pathways	Receptor	Upstream NFL	target	Downstream NFL	target
NF-κB (vertebrate)	TLR4 (innate) TIR (innate) TCR/BCR (adaptive)	A20[1,2]	RIP (outside the nucleus)	IκBα[3]	NF-κB (inside the nucleus)
Imd (invertebrate)	PGRP-LC	Pirk[4]	PGRP-LC (outside the nucleus)	Repressosome complex[5]	NF-κB (inside the nucleus)
Toll (invertebrate)	Toll	NA	NA	Cactus	NF-κB (inside the nucleus)

Jasmonate (plants)	JAT1	CYP94B3[6]	JA-Ile (outside the nucleus)	JAM[7]	MYC (inside the nucleus)

1: (Pujari et al., 2013); 2: (Coornaert et al., 2009) 3: (Zabel & Baeuerle, 1990); 4: (Kleino et al., 2008); 5: (Kim et al., 2007); 6: (Koo et al., 2011); 7: (Sasaki-Sekimoto et al., 2013)

*Here NFLs are listed which are activated upon infection. These differ from negative regulators that control the baseline expression of genes.

Methods

Model simulations

We designed a stochastic model of the Imd pathway, which entails NFLs functioning at different steps of signaling. To assess the robustness of the model and to explore how biological variation in the function of NFLs affects response dynamics, we examined an alternative model featuring a Toll module with a distinct downstream NFL from the one in the Imd model (Fig. 1). We implemented the two models using the Gillespie algorithm (Gillespie, 1976), where reactions capture the change of proteins within an immune signaling pathway. At each step, a reaction is probabilistically chosen and carried out. The likelihood of a reaction depends on its rate relative to the total rates of all reactions. In both models, time is discrete (T = 1,000). The complete models are presented in detail in the supplemental text. We performed all simulations in Python. The code repository is accessible here: https://github.com/danialasg74/Positioning-of-negative-feedback-loops-and-noise/tree/main/codes%20posted%20for%20reveiw

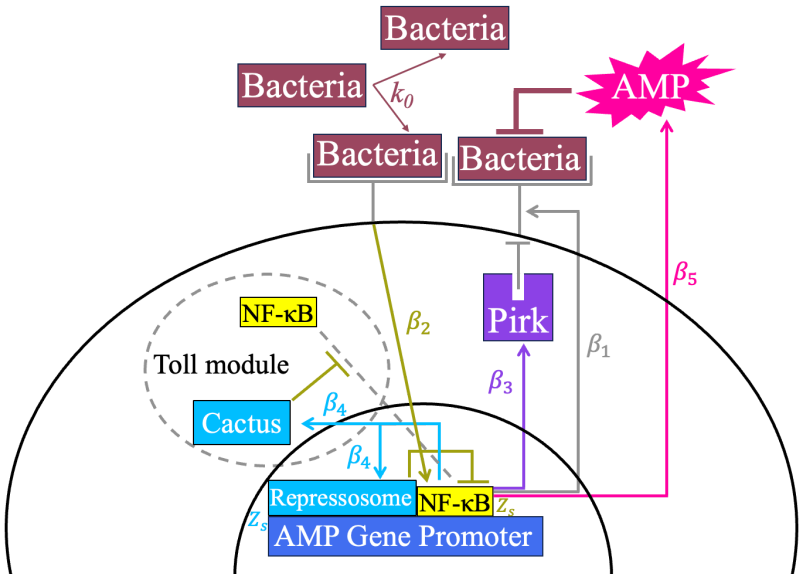

Fig. 1. Simplified diagrams of Imd/Toll pathway. Bacteria proliferate at a rate k_0 and bind to receptors. NF-κB transcription factors are activated by bacteria-receptor complexes at a rate β_2. NF-κB induces the production of receptors (β_1), Pirk, which is the upstream NFL(β_3), Repressosome (in Imd) and Cactus (in Toll), which are the downstream NFLs (β_4), and AMPs (β_5). Pirk reduces receptors in both models. Repressosome competes with NF-κB for binding to the promoter of the AMP gene, and Cactus inhibits the entry of NF-κB to the nucleus. The binding energies of the NF-κB and repressosome are Z_n and Z_s, respectively. The colors of arrows are based on the affected components in each reaction.

Simulation of bacterial encounters

We tested Imd and Toll models in deterministic, stochastic, and periodic environments. In the deterministic environment, we set the initial condition for B (bacteria) to 1 ($B_0 = 1$) (Fig. 2A). In stochastic environments, before calculating the rates of reactions, at each time point t, we set $B(t)$ to either 1 or 0. This is determined by a random walk (T = 1,000 steps) on a 100×100 lattice populated by bacterial colonies. If the host encounters bacteria at time t, $B(t)$ = 1; otherwise $B(t)$ = 0. In stochastic environments, the initial condition for the number of bacteria is set to zero ($B_0 = 0$). We simulated two stochastic environments. In one, 300 bacterial colonies are located next to each other, and in another 200 bacterial colonies are scattered in the environment (Fig. 2B). The uniform environment has fewer bacteria to compensate for the higher frequency of host-bacteria encounters in the uniform environment compared to the patchy environment. Finally, we simulated periodic bacterial encounters that happen at different time intervals (I = 200, 100, 20, 5, and 1). If I = 5, the host encounters bacteria every five steps (Fig. 2C), whereas, if I = 1, the host encounters bacteria at every step. We simulated host-pathogen encounters following Asgari et al., 2023.

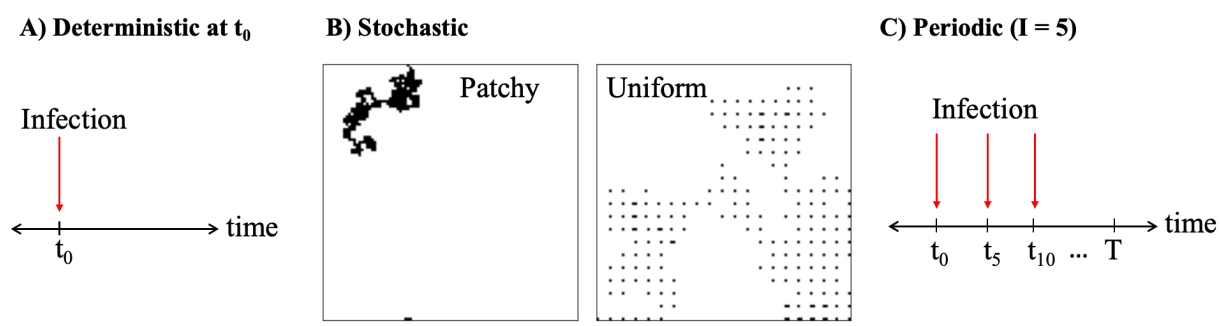

Fig. 2. Simulations of host-pathogen encounters. Panel A shows a deterministic encounter at t_0. Panel B shows stochastic encounters. Each dot represents a bacterial colony on a 100×100 lattice. In panel C, a periodic encounter at every five steps (I = 5) is shown.

Parameter regimes

We considered four parameter regimes to examine the effect of NFLs on the dynamics of Imd and Toll models (Table 2). The four regimes are based on two parameters that specify the production rate of NFLs (Pirk and repressosome/Cactus). The upstream (β_3) and downstream (β_4) NFLs can either be weakly expressed (β_3 or $\beta_4 = 1$) or strongly expressed (β_3 or $\beta_4 = 10$). We chose these values based on the qualitative assessments of several simulations before conducting the systematic analysis of the models.

There are four background parameters ($\beta_1, \beta_2, \beta_5, \lambda$) in models of Toll and Imd. These are distinct from parameters that determine the dynamics of NFLs (β_3, β_4, Z_s) and the binding energy of NF-κB (Z_n). For each of the background parameters, we considered 10 different values (ranging from 1 to 10 for $\beta_1, \beta_2, \beta_5$ and from 0.1 to 1 for λ). We explored the behavior of the system across all combinations of background parameter values. This constitutes a total of 10,000 parameter combinations for each of the four regimes ($10^4 = 10,000$). In the Imd model, across all parameter regimes, we maintained the following condition: $Z_s = Z_n = 1$. This ensures that the binding of the repressosome is only influenced by the relative number of NF-κB to repressosome, which in turn is influenced by β_4 that varies across the four regimes. For consistency in the Toll model we set $Z_n = 1$.

In the Imd model, in addition to the production rate of the repressosome, the repressosome binding energy (Z_s) affects the strength of the downstream NFL. Therefore, we also conducted simulations where we varied the value of Z_s while keeping Z_n fixed at 1.

Table 2. Four parameter regimes with different strengths of NFLs

Effect	β_3(Production rate of Pirk)	β_4(Production rate of repressosome/Cactus)
Both weak	1	1
Upstream biased	10	1
Downstream biased	1	10
Both strong	10	10

Calculation of noise, average AMP expression, and fitness

We quantified noise in AMP expression at every time point (T = 1,000) by calculating the coefficient of variation (Wang & Zhang, 2011) for each parameter set (a total of 40,000

parameter sets across four regimes) across 10,000 replicate simulations (Eq. 1). We then determined the magnitude of the noise by calculating the arithmetic average of the noise across all time points (Eq. 2). We measured the average AMP expression for each parameter set by calculating the arithmetic mean of average AMP expression across all time points (T = 1,000) for 10,000 replicates simulations (Eq. 3).

$$CV_t = \frac{\sigma_t}{\mu_t} \tag{1}$$

If $\mu_t = \sigma_t = 0$, CV_t is set to 0.

$$Noise = \frac{\sum_{t=0}^{T} CV_t}{T} \tag{2}$$

$$\bar{AMP} = \frac{\sum_{t=0}^{T} \mu_t}{T} \tag{3}$$

We measured fitness by calculating the average number of immune proteins and the average pathogen load ($\overline{B}$) during the host lifetime (T = 1,000). We assumed that fitness is exponentially reduced as pathogen load and the number of immune proteins increases (due to immunopathology or energetic costs). We used three fitness functions (Eq. 4-6) that vary in terms of how they weigh the relative contributions of immune protein production and pathogen load on the reduction of fitness.

In Eq. 4, pathogen load (*B*) and production of all immune proteins (*R*, *N*, *P*, *S*, and *A*) impose costs:

$$F = e^{-(\bar{B}+\bar{R}+\bar{N}+\bar{P}+\bar{S}+\bar{A})} \tag{4}$$

For the model of the Toll pathway, N (NF-κB) in Eq. 4 is replaced by No (supplemental text). This is because we assume that the entry of NF-κB into the nucleus ($No \rightarrow N$) is not costly, and the cost is only due to the activation of NF-κB.

In Eq. 5, the pathogen load and production of AMP (*A*) impose costs due to immunopathologic effects of AMP expression, but we assume (unlike in Eq. 4) that energetic costs associated with the production of signaling proteins are negligible:

$$F = e^{-(\bar{B}+\bar{A})} \tag{5}$$

In Eq. 6, only the pathogen load imposes costs, as would be true when the cost of the immune response is negligible compared to the deleterious effects of bacteria:

$$F = e^{-(\bar{B})} \tag{6}$$

Results

Upstream NFLs decrease noise whereas downstream NFLs increase noise in AMP expression

First, we examined how the relative strength of NFLs that are located downstream or upstream of the pathway can impact noise in AMP expression. We manipulated noise by changing the background parameter values of the models across four parameter regimes (Table 2). We found that regardless of the bacterial distribution and proliferation rate, across both Imd and Toll models, parameter sets with larger noise values were generally observed for the "downstream biased" regime (cyan density plots in Fig. 3). We confirmed this by quantifying noise following the reduction of the binding energy (Z_s in reaction 14 of the supplemental text) of the repressosome (more efficient binding) when both NFLs were weakly expressed. Reducing the binding energy removed smaller noise values from the distribution, confirming that a strong downstream NFL increases the noise in AMP expression (Fig. S3). In the "upstream biased" regime (purple density plots in Fig. 3), noise distributions were shifted toward smaller values. Thus, an upstream NFL such as Pirk reduces noise in AMP expression. We found that regardless of the model, parameter regime, environmental context, and bacterial proliferation rate, the higher noise values were generally associated with characteristic background parameters: a low AMP production rate and high degradation rate of proteins (Fig. S4, 5, and 6).

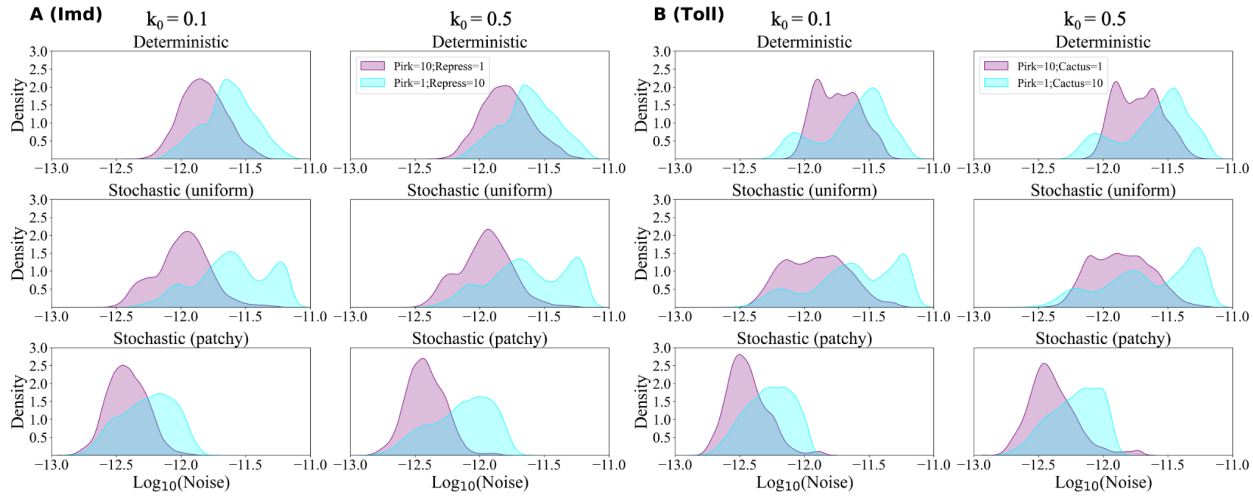

Fig. 3. Density plots for the distribution of noise across the two regimes (strong upstream NFL = purple, strong downstream NFL = cyan) for models of Imd and Toll. The Y-axis is the density of the distribution and the X-axis represents the $\log_{10}$ transformed values of noise. The variation in noise observed in each graph arises from different combinations of background parameter values. The plots on the same column have the same bacterial proliferation rate ($k_0 = 0.1$ and $k_0 = 0.5$) and plots on the same row same distribution of bacteria (A deterministic encounter at t_0, and two stochastic environments).

Noise is beneficial upon repeated encounters with pathogens if the immune response is cheap

Next, we examined how different levels of noise in AMP expression (due to variations in background parameter values) influence fitness while controlling for the average AMP expression (Eq. 3). This prevents confounding the impact of noise and the magnitude of AMP expression (average AMP) on fitness. This is important because the coefficient of variation, which is the measure of noise, is inversely proportional to the average expression (Eq. 1). We did not find noise in AMP expression to be beneficial under a deterministic encounter at t_0 or under stochastic encounters in patchy environments (Fig. S7 and 8). On the other hand, we found a benefit for noisy AMP expression when bacteria are uniformly distributed (Fig. S9). However, this was dependent upon the fitness function.

In uniform environments, parameter values with higher noise have higher fitness when the immune response is cheap. This happens if only the production of AMPs (immunopathology only; Eq. 5) and not all immune proteins (energetic costs, Eq. 4) incur a cost or when the cost of immune response is negligible compared to the damage caused by bacteria (Eq. 6). If AMP production is costly (Eq. 5), parameter values with higher noise have higher fitness when AMP expression is low because too much AMP (high average expression) reduces fitness, masking potential benefits of noise. When there is no cost of immunity (Eq. 6), noise is beneficial regardless of the expression level. This was observed for all four parameter regimes across both models and proliferation rates (Fig. S9). The results for the "downstream biased" regime of the

Imd model are shown in Fig. 4. When the immune response is cheap (Fig. 4, two panels on the right), parameter sets with low levels of AMP expression and high noise have higher fitness.

The high frequency of exposure in uniform environments might explain the benefit of noisy AMP expression. To investigate this, we considered periodic bacterial encounters with varying time gaps (I = 200, 100, 20, 5, 1) for the Imd model. We tested this for the "downstream biased" regime because it has higher noise in AMP expression compared to other regimes (Fig. 3). We found similar results to the uniform environment, except when I = 1 (Fig. S10). This suggests repeated encounters, whether stochastic or deterministic, favor noise in AMP expression. High noise levels may prevent complete AMP expression shutdown following induction, leading us to hypothesize that residual AMP from prior encounters increases survival upon subsequent ones. Indeed, higher noise levels result in AMP expression resembling a constitutive defense, while lower levels resemble an induced response (Fig. S11). By adding a baseline level of AMP (δ in reaction 14) to the model of Imd, the benefit of noise disappeared ($\delta = 0.1$; Fig. S12). This confirms that noise confers a benefit by shifting the response from a strictly induced to a more consistent expression of AMP.

To confirm the benefit of noise independent of parameter values, we compared the fitness of a stochastic Imd model (Gillespie) to a deterministic one (ODE). This was done under both a deterministic bacterial encounter at t_0 and periodic encounters. First, we divided parameter sets into optimal and sub-optimal based on the fitness values of the deterministic ODE model (Fig. 5). Thus, the optimality of a parameter set is its performance in the absence of noise. In the stochastic model, upon periodic encounters, optimal parameters with high noise have high fitness, while optimal parameters with low noise perform poorly (Fig. 5 A and B). Therefore, during periodic encounters, noise outweighs optimality in determining fitness, and fitness is higher for scenarios with higher noise in AMP expression. Conversely, in the stochastic model, upon a deterministic encounter at t_0, optimal parameter sets have high fitness regardless of noise, with lower noise levels resulting in even higher fitness (Fig. 5 C and D). Thus, for a deterministic encounter at t_0, optimality outweighs noise in determining fitness.

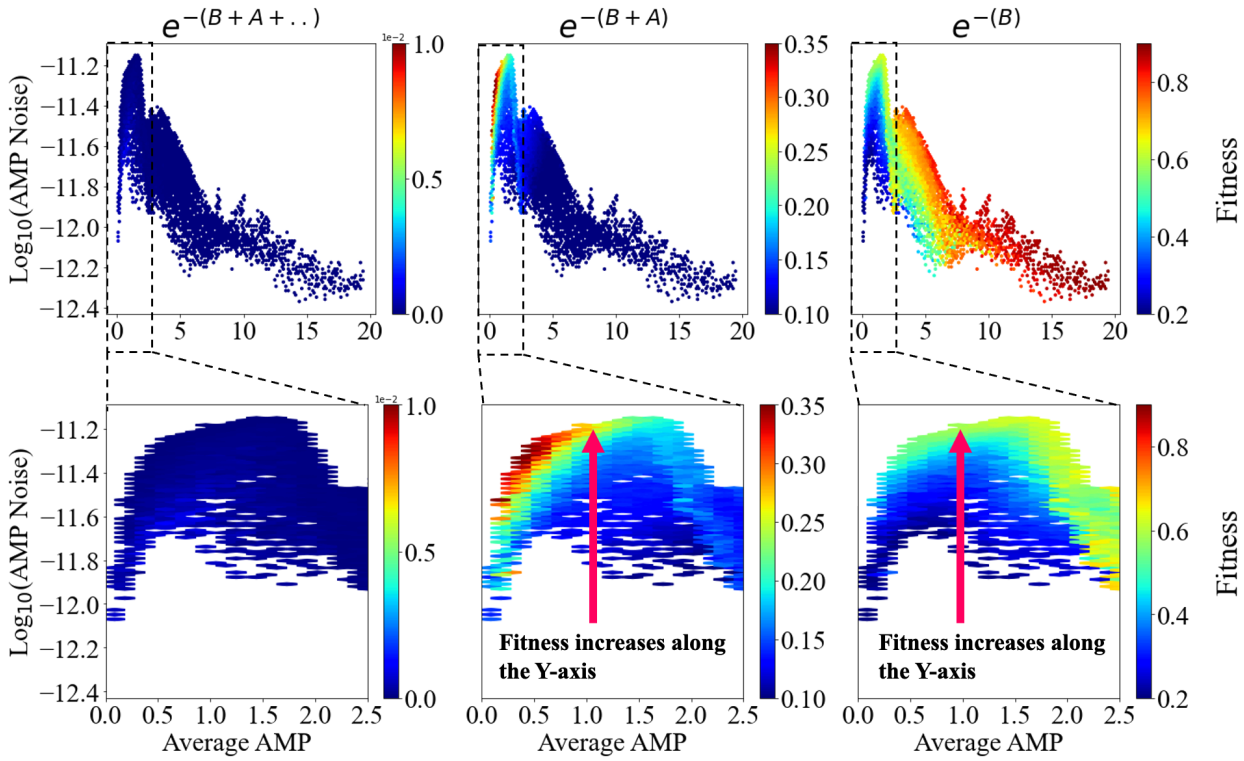

Fig. 4. Effect of noise in AMP expression on fitness for induced defenses with different average AMP expression. The fitness is measured using three different functions (columns). The fitness values (color of the heatmap) for the "downstream biased" regime in the Imd model are plotted for 10,000 combinations of background parameter values, which are associated with different noise values (Y-axis) and different average AMP expressions (X-axis). The results are shown for encounters with bacteria with uniform distribution and a low proliferation rate ($k_0 = 0.1$).

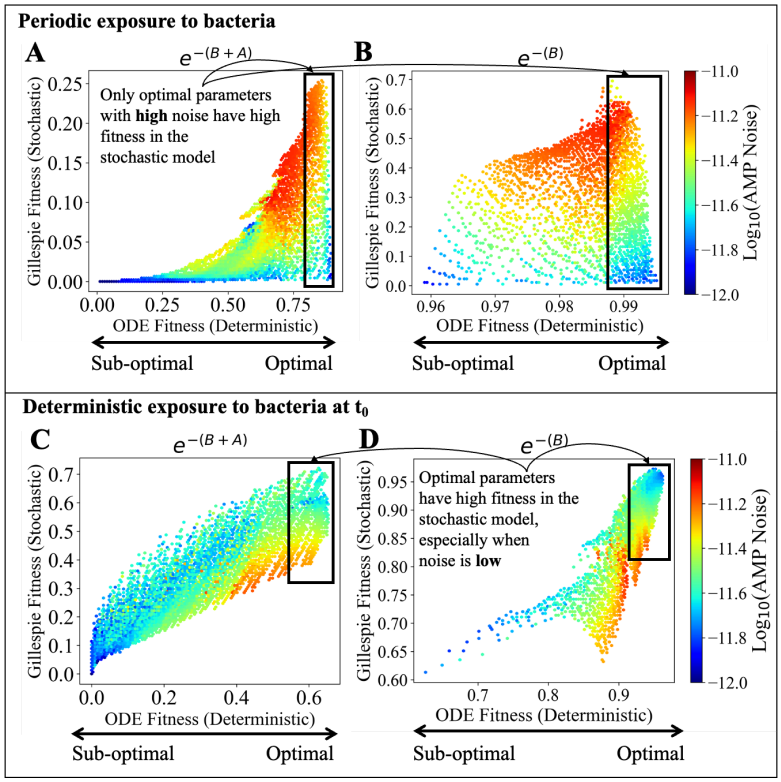

Fig. 5. Fitness values of the Imd model derived from Gillespie simulations (Y-axis) and the numerical solution of ODE (X-axis) are plotted for 10,000 combinations of background parameters (data points) with different noise levels (color). This is done for periodic encounters (I = 200) (A and B) and a deterministic at t_0 (C and D) with bacteria ($k_0 = 0.1$) for the "downstream biased" regime. The parameter combinations are divided into optimal and sub-optimal based on the fitness value of the deterministic Imd. For ($F = e^{-(B)}$), only parameters with an average AMP < 2 are plotted to clearly show the benefit of noise and distinguish it from the benefit of high AMP expression.

Downstream NFLs optimize induction by noisy AMP expression

To tease apart the role of upstream and downstream NFLs on noise regulation and fitness, we compared the "upstream biased" with the "downstream biased" regime. We focus on uniform environments because noise is beneficial in such environments (Fig. 4). The "downstream biased" regime outperforms the "upstream biased" regime for parameters with higher noise. On the other hand, the "downstream biased" regime underperforms at low noise for the low bacterial proliferation rate ($k_0 = 0.1$) (Fig. 6 B and F). For a higher bacterial proliferation rate ($k_0 = 0.5$), the "downstream biased" outperforms the other regime regardless of the noise level (Fig. 6 D and H).

Next, we asked whether fitness differences were due to the reduction of noise by the upstream NFL (Pirk) or due to an increase in noise by downstream NFLs (Cactus or repressosome) (Fig. 3). To test this, we compared the fitness of the "downstream biased" regime

with “both weak” and “both strong” regimes (Fig. 7). Fitness and noise differences between “downstream biased” and “both weak” regimes were not significant and slight differences were not due to differences in noise levels (Fig. 7 A and B). This suggests that the absence of an upstream NFL results in higher noise and fitness values. On the other hand, fitness values for the “both strong” regime were larger than the “upstream biased” regime, and some parameter combinations in the “both strong” regime have high noise and high fitness similar to the “downstream biased” regime, especially in the Imd model (Fig. 7 C and D). Therefore, while the upstream NFL (Pirk) reduces noise and fitness in environments with uniform bacterial distributions, its impact can be mitigated by a strong downstream NFL (such as repressosome or Cactus).

We have identified two unique benefits associated with the "upstream biased" regime. First, in the “upstream biased” regime, fitness is robust to changes in background parameter values (i.e., across genotypes), as shown by purple density plots in Fig. 6 and gold density plots in Fig. 7. This can be advantageous because a low fitness variance increases geometric fitness across generations when the host experiences fluctuating conditions. In addition, mutations in parameters have potentially fewer deleterious effects on fitness. Finally, if maintaining an immune response is expensive (Eq. 4), the “upstream biased” regime performs better than the “downstream biased” regime because it reduces the cost of response (Fig. S13).

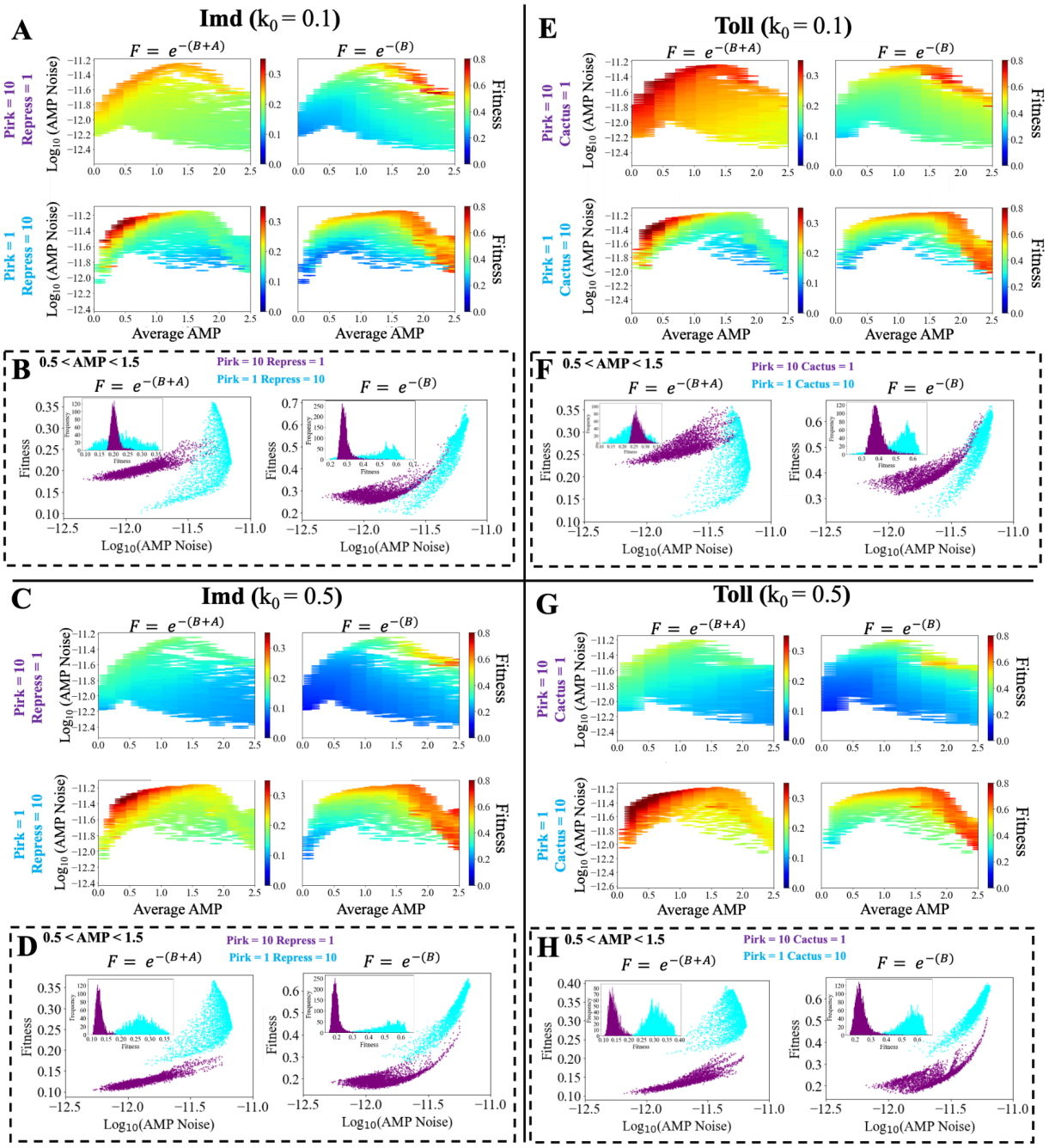

Fig. 6. The relationship between noise and fitness when average AMP expression is low for "downstream biased" and "upstream biased" regimes In the Imd (A, B, C, and D) and Toll (E, F, G, and H) models. The heatmaps (A, E, C, and G), show fitness for different AMP noise values when 0 < average AMP < 2.5. The graphs in panels B, D, F, and H show changes in fitness values (Y-axis) for background parameter values with different noise in AMP expression (X-axis). The small histograms show the fitness distribution across the two regimes. The analysis was performed for two fitness functions ($F = e^{-(B+A)}$ or $e^{-(B)}$).

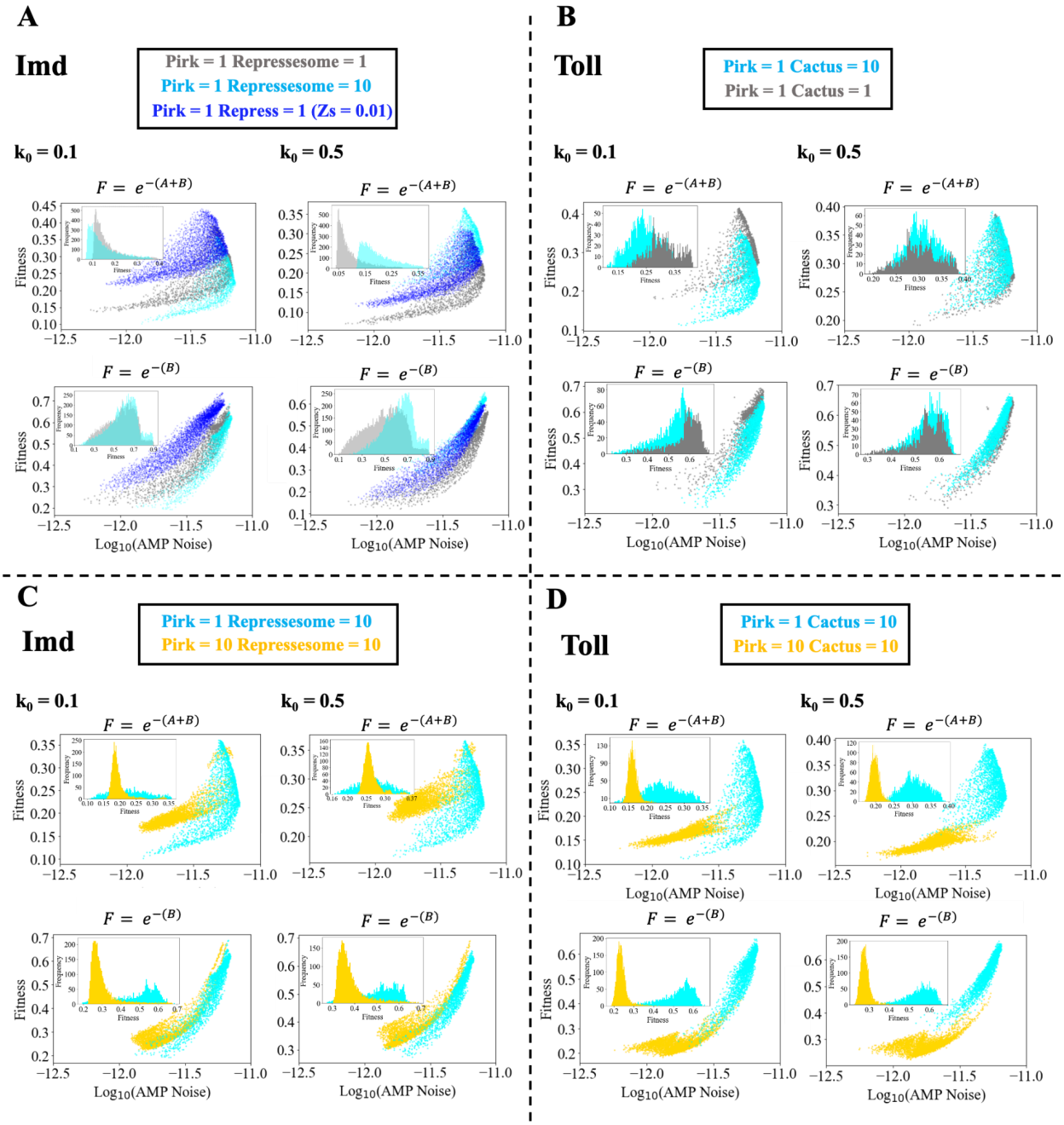

Fig.7. Fitness values (Y-axis) for background parameters with different noise in AMP expression (X-axis) when 0.5 < average AMP < 1.5. The first two panels (A and B) compare the "downstream biased" regime (cyan and blue) to the "both weak" regime (gray). The two panels on the bottom (C and D) compare the "downstream biased"(cyan) regime to the "both strong" regime (gold). The small histograms show the distribution of fitness values across two regimes. The analysis was performed for two fitness functions (rows).

Discussion

We found that the cellular location where NFLs function influences noise in AMP expression and, in turn, host fitness. Specifically, NFLs functioning inside the nucleus increase noise in AMP expression, which is beneficial during periodic exposure to pathogens. On the other hand, NFLs closer to receptors reduce the cost of defense and fitness differences among genotypes. Thus, our model identifies selective forces that shape the relative activity of NFLs at different cellular locations. For example, organisms with frequent exposure to pathogens are expected to increase the activity of NFLs inside the nucleus to increase noise in the expression of immune genes. On the other hand, organisms facing environmental uncertainty might increase the activity of NFLs functioning near receptors to decrease fitness variance across genotypes to improve geometric fitness across generations (bet-hedging).

Repeated host-bacteria encounters favor noise in AMP expression

We found that noise is beneficial upon repeated encounters with bacteria. Repeated exposure to pathogens favors noisy AMP expression, whether due to stochastic or periodic encounters. The theory of stochastic resonance predicts that in sub-optimal non-linear systems, noise improves signal processing (McDonnell & Abbott, 2009) (Fig. S1). This phenomenon was initially observed and extensively studied in response to periodic inputs, and has since been extended to other types of inputs (Collins et al., 1995; Fauve & Heslot, 1983; Jung, 1993). We suggest that stochastic resonance might explain why noise is favored upon repeated encounters with pathogens. Both the Imd and Toll models studied here, along with nearly all immune signaling pathways, are non-linear systems that are regulated by NFLs. In our models, noise is favored when AMP expression is low, which is a sub-optimal response to pathogens. Thus, the observed benefit of noisy AMP expression meets all requirements for stochastic resonance: non-linearity, sub-optimality, and periodic input. Our study does not directly test for optimality of information transfer in noisy signaling pathways with multiple NFLs. Future theoretical investigations could employ information theory to directly test for stochastic resonance in signaling pathways.

An alternative explanation (but not necessarily mutually exclusive) is that noise shifts the dynamics of AMP expression from a purely induced to a more constitutive state. Therefore, noise is favored because it prevents a complete shutdown after initial exposure, improving survival upon subsequent exposure to pathogens. The observation that constitutive AMP expression negates the benefit of noise supports this hypothesis. We propose that noisy expression of immune genes at a low level improves host fitness upon repeated encounters with pathogens and eliminates the need for a costly constitutive expression of immune genes, resulting in a more cost-effective strategy for adapting to microbial environments.

Overall we showed that noisy expression in immune genes is favored when the host induces immune genes at a low level, lacks constitutive expression from other immune genes to complement the action of induced genes, and is consistently exposed to pathogens. This provides

a framework for the identification of immune genes with noisy expression across different taxa. The utilization of induced and constitutive defenses varies across species and host tissues (Asgari et al., 2022; Imler & Bulet, 2005; Kaiser & Diamond, 2000; Thomma et al., 2002; Yamasaki & Gallo, 2008). Moreover, variations in life history and ecology result in differences in the degree of exposure to pathogens (Horrocks et al., 2011). Our results highlight the significance of accounting for such variations to understand the benefits of the noisy expression of immune genes. Specifically, we predict that the prevalence of noisy expression in immune genes would be more pronounced in organisms that heavily rely on induced, rather than constitutive defenses, and are consistently exposed to pathogens throughout their lifespan. Such insights can help with future empirical analyses to understand the impact of noise on the optimal response of signaling pathways.

Effect of upstream and downstream NFLs on noise and fitness

We found that downstream NFLs increase noise in AMP, which is beneficial upon repeated exposure to pathogens. Consistent with Asgari et al. 2023, we found that, when exposed to rapidly dividing bacteria, downstream NFLs increase fitness more than upstream ones, regardless of noise. Furthermore, we showed that noise becomes a distinguishing factor in the benefits of NFLs operating at different stages of signaling when the bacterial proliferation rate is low. On the other hand, upstream NFLs reduce noise in AMP expression, decrease fitness variation among genotypes, and mitigate the overall cost of immunity. We observed a combined effect of two NFLs when both upstream and downstream NFLs were highly expressed. Specifically, upon high expression of both NFLs, fitness variation across genotypes is reduced and some genotypes have high noise in AMP expression and high fitness upon repeated encounters with pathogens.

While we focus on insect signaling, signaling pathways in other species share structural paradigms and provide support for the generality of our results across taxa. For example, Cheong et al., (2011) showed that A20, an upstream NFL in vertebrate NF-κB signaling, reduces noise in NF-κB activity, similar to the noise reduction observed in AMP expression with high Pirk expression in our models. We also observed that a higher expression of upstream NFLs decreases variation in fitness among individuals with diverse parameters governing immune response regulation (i.e., different genotypes). This can be beneficial in fluctuating environments, where no single genotype exhibits consistently high fitness across all generations. Therefore, high expression of upstream NFLs may lead to the emergence of suboptimal defenses characterized by low fitness variation. This increases geometric fitness across generations, a phenomenon commonly referred to as conservative bet-hedging (Childs et al., 2010). Further empirical studies are required to elucidate the role of upstream NFLs in conservative bet-hedging under uncertain environmental conditions.

We found that high expression of downstream NFLs generates noisy expression of AMPs, which confers benefits upon repeated encounters with pathogens. Previous studies on vertebrate NF-κB signaling identified oscillation of NF-kB between cytoplasm and nucleus in

response to periodic stimulations (Tay et al., 2010). The Oscillation of NF-κB is important for the synchronized regulation of a larger number of genes, which are activated by NF-κB (Zambrano et al., 2016). These oscillations are caused by the downstream NFL (IκBα), which functions within the nucleus and negatively regulates NF-κB, and are facilitated by noise within the signaling pathway (Heltberg et al., 2016; Kellogg & Tay, 2015). Our study highlights the advantages of noisy AMP expression during repeated pathogen exposure and illuminates further crucial aspects of downstream NFLs and noise in optimizing responses.

Here we used simple models of immune signaling to analyze the effect of multi-level regulation on noise and fitness. Future theoretical investigations could employ more detailed models of immune signaling or other pathways to validate these insights in specific systems. Nonetheless, our study fills a gap in research by investigating the impact of NFLs operating at various stages of signaling on the noisy expression of target genes and its ultimate influence on host fitness. The broader applicability of our findings extends beyond immune signaling pathways, as numerous biological pathways are regulated by multiple NFLs that function at different stages of signaling.

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