# Quantifying Multi-Signal Quorum Sensing Clarifies Bacterial Responses to Environmental Variation

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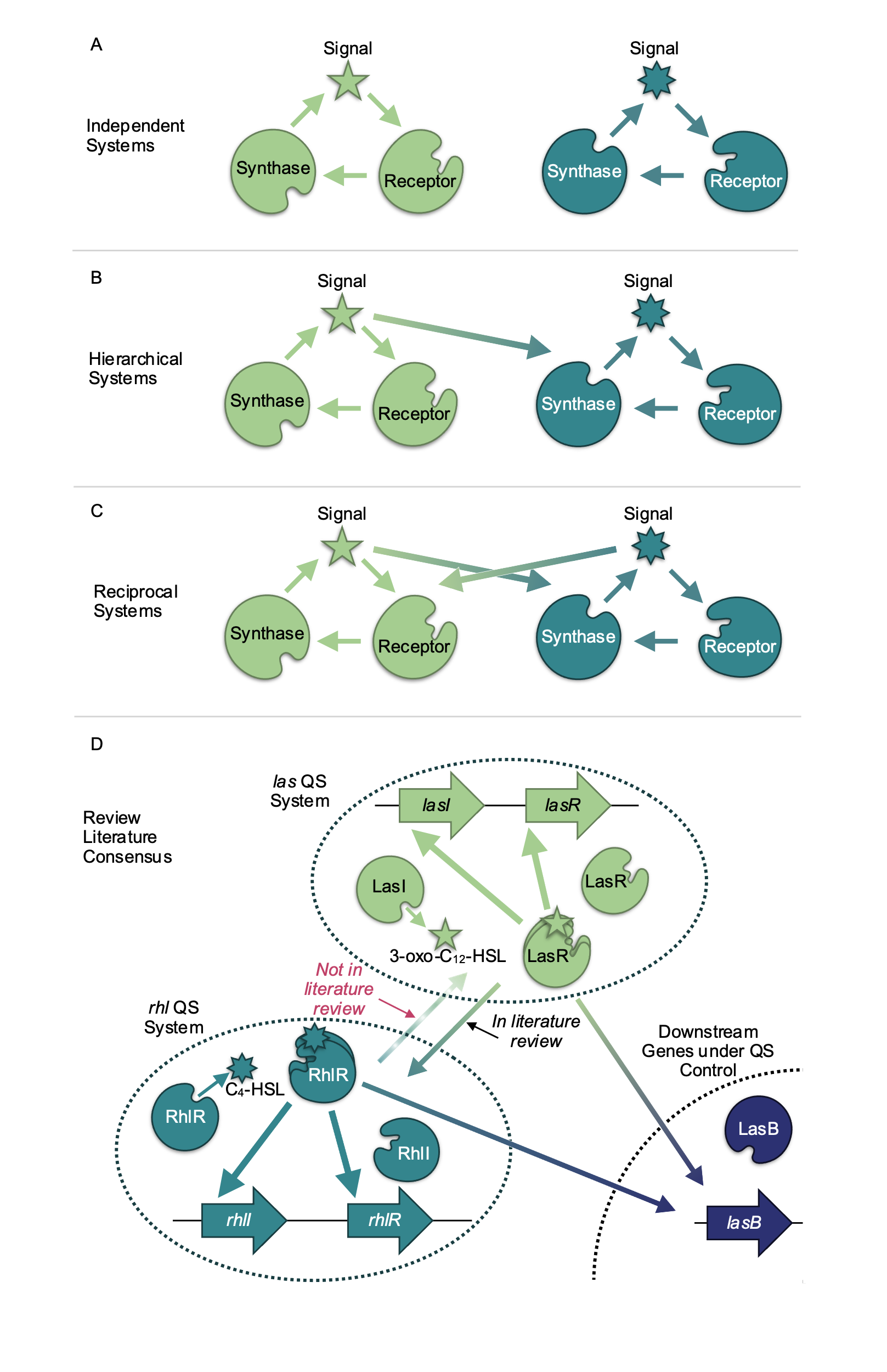
**Abstract:** Bacterial quorum sensing is often mediated by multiple signaling systems that interact with each other. The quorum sensing systems of *Pseudomonas aeruginosa,* for example, have been considered hierarchical, with the *las* system acting as a master regulator. By experimentally controlling the concentration of auto-inducer signals in a signal deficient strain (PAO1Δ*lasI*Δ*rhlI*), we show that the two primary quorum sensing systems—*las* and *rhl*—act reciprocally rather than hierarchically. Just as the *las* system’s 3‑oxo‑C12‑HSL can induce increased expression of *rhlI,* the *rhl* system’s C4‑HSL increases the expression level of *lasI.* We develop a mathematical model to quantify relationships both within and between the *las* and *rhl* quorum sensing systems and the downstream genes they influence. The results show that not only do the systems interact in a reciprocal manner, but they do so asymmetrically, cooperatively, and nonlinearly, with the combination of C4‑HSL and 3‑oxo‑C12‑HSL increasing expression level far more than the sum of their individual effects. We next extend our parameterized mathematical model to generate quantitative predictions on how a QS-controlled effector gene (*lasB*) responds to changes in bacterial stationary phase density, and find close quantitative agreement with an independent dataset. Finally, we use our parameterized model to assess how changes in multi-signal interactions modulate functional responses to variation in social (population density) and physical (mass transfer) environment and demonstrate that a reciprocal architecture is more responsive to density and more robust to mass transfer than a strict hierarchy.

## Introduction

WIthin many bacterial species, cells communicate with each other by exchanging diffusible signal molecules (Papenfort and Bassler 2016; Whiteley, Diggle, and Greenberg 2017). This mechanism, known as quorum sensing (QS), has been well-studied at the level of specific molecular interactions. We now understand how those molecular interactions shape the creation of and response to signal molecules in model organisms such as *Pseudomonas aeruginosa* (Pearson, Pesci, and Iglewski 1997). We have identified downstream effector genes such as virulence factors whose production depends on QS signals (Chugani et al. 2001; Kiratisin, Tucker, and Passador 2002), and we have recognized that many species possess multiple QS circuits (Papenfort and Bassler 2016). Despite this knowledge, we face gaps in our understanding of how quorum sensing influences bacterial behavior. How does QS quantitatively guide bacterial actions in response to defined environmental conditions? What benefits do multiple QS circuits provide? And ultimately, how does QS contribute to bacterial fitness? Answering these questions requires an understanding of quorum sensing at the dynamical systems level as well as the molecular level.

Quorum sensing relies on several components interacting in a dynamical system (Popat et al. 2015; Pérez-Velázquez and Hense 2018). Individual cells synthesize small molecules called signals or inducers. These diffuse or are actively transported between the intracellular and extracellular environments (Pearson, Van Delden, and Iglewski 1999). Within cells, signal molecules bind to receptor proteins that serve as transcription factors (Bottomley et al. 2007). As signal concentration grows, genes activated by these transcription factors trigger a change in the cell's behavior (Whiteley, Lee, and Greenberg 1999). Those molecules related to a particular signal—the signal synthase, the signal molecule, and the cognate receptor—form a quorum sensing system. Some bacterial species have multiple QS systems, the opportunistic pathogen *Pseudomonas aeruginosa* among them. Among its multiple systems, *las* and *rhl* acyl-homoserine lactone (AHL) signaling systems have been especially well studied (Pesci et al. 1997; Lee and Zhang 2015). The *las* system includes the LasI signal synthase, N-(3-oxododecanoyl)-l-homoserine lactone (3‑oxo‑C12‑HSL) signal, and LasR receptor. The corresponding components of the *rhl* system are RhlI, N-butyryl-homoserine lactone (C4‑HSL), and RhlR. Schuster and Greenberg (2007) estimate that these two systems control expression of as much as 10% of the bacterial genome.

*P. aeruginosa* provides a model for understanding interactions between multiple QS systems. How does the behavior of one system, determined by the concentration of signal it produces, affect the behavior of a different system? How does expression of the one system’s synthase or receptor respond to the concentration of another systems signal? We classify possible multi-system architectures into three broad patterns shown in Figure 1. *Independent systems* (Figure 1A) have no influence on each other; *hierarchical systems* (Figure 1B) have a relationship but only in one direction, and *reciprocal systems* (Figure 1C) each exert influence on the other. At this level we do not consider the underlying mechanism(s) of the inter-system effects. For example, the signal of one system may bind directly to the receptor of the other; alternatively, the signal/receptor complex of one system may act as a transcriptional regulator of components in the second system. In both cases we simply denote the first system as influencing the second.



**Figure 1. The P. aeruginosa QS regulatory network is typically viewed as a hierarchy with the las system on top.** In general, the regulatory network of two QS systems may be organized in three architectures: (A) Independent, in which the signal of each has no influence on the expression of synthase or receptor in the other. (B) Hierarchical, where one system’s signal influences expression of the other’s components, but without reciprocation. And (C) reciprocal, where both systems’ signals influence the others’ components. The consensus of the review literature for P. aeruginosa (D) as summarized in 17 review papers published since 1996 (Tables S.1 and S.2), suggests a hierarchical architecture for the las and rhl systems. All papers show the las system affecting the rhl system, but none identify a las synthase or receptor gene as a target of the rhl system.

In the case of *las* and *rhl,* independent, isolated operation was eliminated as early as 1996 when Latifi et al. (1996) used *lacZ* transcriptional fusions to show that the combination of LasR and 3‑oxo‑C12‑HSL controls expression of *rhlR,* demonstrating that the *las* system influences the *rhl* system. These and other results have led many researchers to view *las* and *rhl* as a hierarchy, with the *las* system serving as master QS system controlling both its own activation and that of the *rhl* system (Figure 1D). We confirm this consensus perspective via a structured literature review of review articles (Tables S.1 and S.2). While the review literature is silent on whether the *rhl* signal C4‑HSL can influence the expression of the *las* synthase or receptor, several studies demonstrate that the *rhl* system can activate even when the “top of the hierarchy” *lasR* gene is deleted (Dekimpe and Déziel 2009), indicating limitations of the simple hiearchy model. If *lasI* or *lasR* respond to the *rhl* signal, then the strict hierarchical view may be missing an important factor that determines the overall system response.

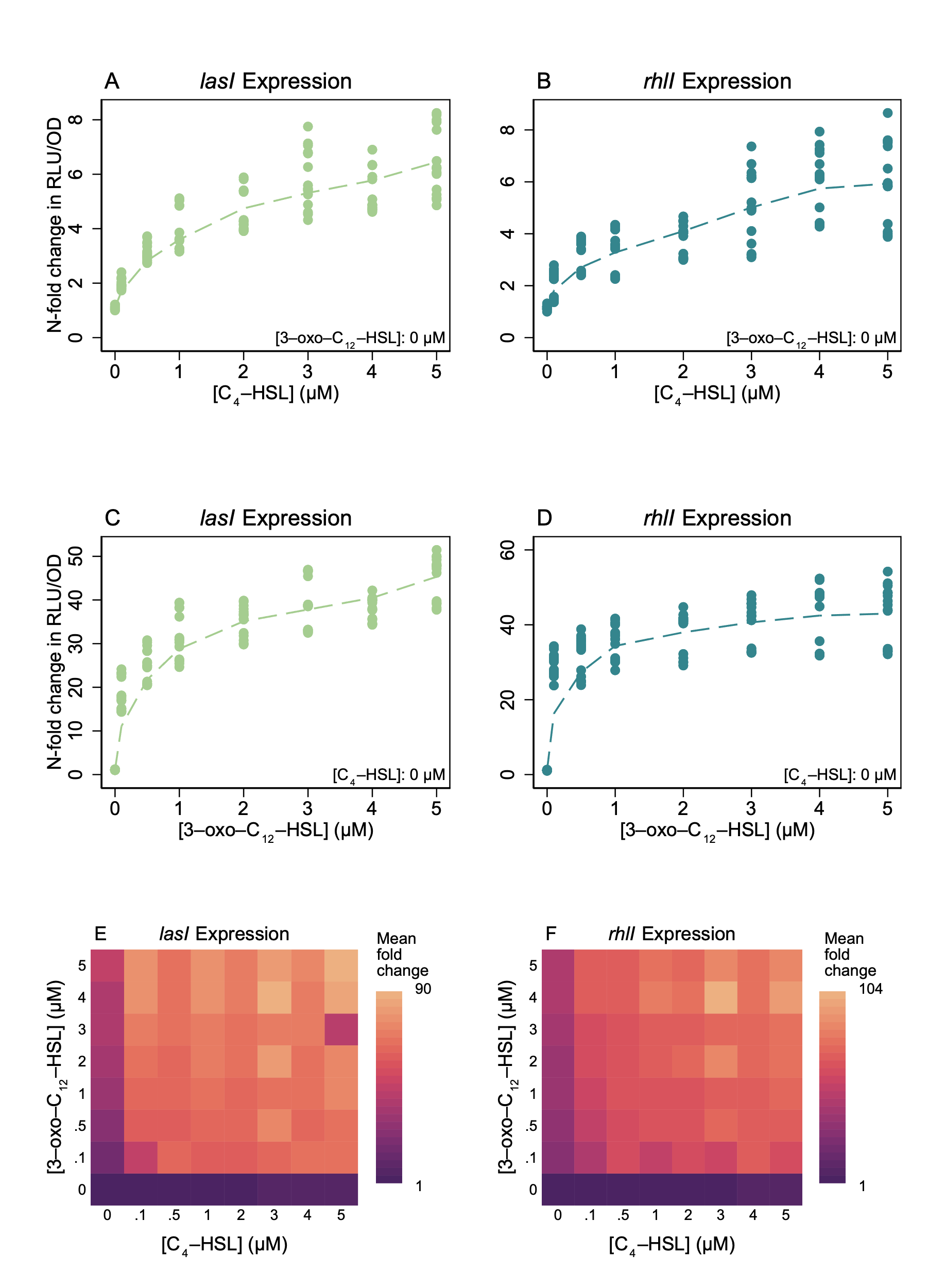
Our experiments explicitly examine the influence of both QS systems on each other, and the resulting data reveal three key results. First, the traditional hierarchical view of *las* and *rhl* is incomplete. Our results confirm that *las* can exert control over the *rhl* system, but we also observe the converse: *rhl* substantially influences the *las* system, specifically expression of *lasI.* Second, we use our data to parameterize a new mathematical model of multi-signal regulatory dynamics, producing quantitative estimates on how signal modules influence each other, and the expression of downstream effector genes. Finally, we embed our multi-signal model in an explicit environmental context, to produce quantitative predictions on how QS-controlled behaviors change with changing physical and social conditions. Specifically, we show that, compared to a strict hierarchy, the reciprocity we observe provides more sensitivity to population density and more robustness to interfering physical environmental variation.

## Results

To uncover interactions between the *las* and *rhl* systems, we experimentally assess QS gene expression in an AHL null strain (PAO1Δ*lasI*Δ*rhlI*) exposed to defined, exogenous concentrations of the signal molecules 3‑oxo‑C12‑HSL (*las* system) and C4‑HSL (*rhl* system). We use bioluminescence (lux) reporters for *lasI* and *rhlI* to estimate expression levels of the respective signal synthase genes. We then develop mathematical models to quantify the effects of each system on the other and their consequent responses to environmental variation.

### The *las* and *rhl* Systems Interact Reciprocally

We first evaluate quorum sensing behavior under the influence of a single signal. We establish a baseline expression level by measuring reporter luminescence with no signal present. We then observe the increase in luminescence as exogenously controlled signal concentration increases. The ratio of luminescence with signal to luminescence with no signal represents the fold-change in expression induced by the defined signal concentration. Figure 2A,B shows the results for 3‑oxo‑C12‑HSL. As expected, expression of both genes increases as signal concentration increases. The availability of the *las* signal molecule influences the expression of *rhlI* as well as *lasI,* and, therefore, the *las* system affects the *rhl* system.



**Figure 2. Both the las signal 3‑oxo‑C12‑HSL and the rhl signal C4‑HSL increase the expression of lasI and rhlI in a signal null PAO1.** (A,B) Effect of 3‑oxo‑C12‑HSL alone. (C,D) Effect of C4‑HSL alone. (E,F) Effect of both signals together. Plots show fold-change in RLU/OD (relative light units per optical density) values compared to baseline with no exogenous signals in NPAO1∆lasI∆rhlI cultures. Genomic reporter fusions lasI:luxCDABE, rhlI:luxCDABE, and lasB:luxCDABE were used to generate luminescence. Points are individual observations within the time window of peak expression; dashed lines show fitted Michaelis-Menten functions (Methods, Table S3). Figures S.1 and S.2 show the underlying expression data for the entire time course of the experiments.

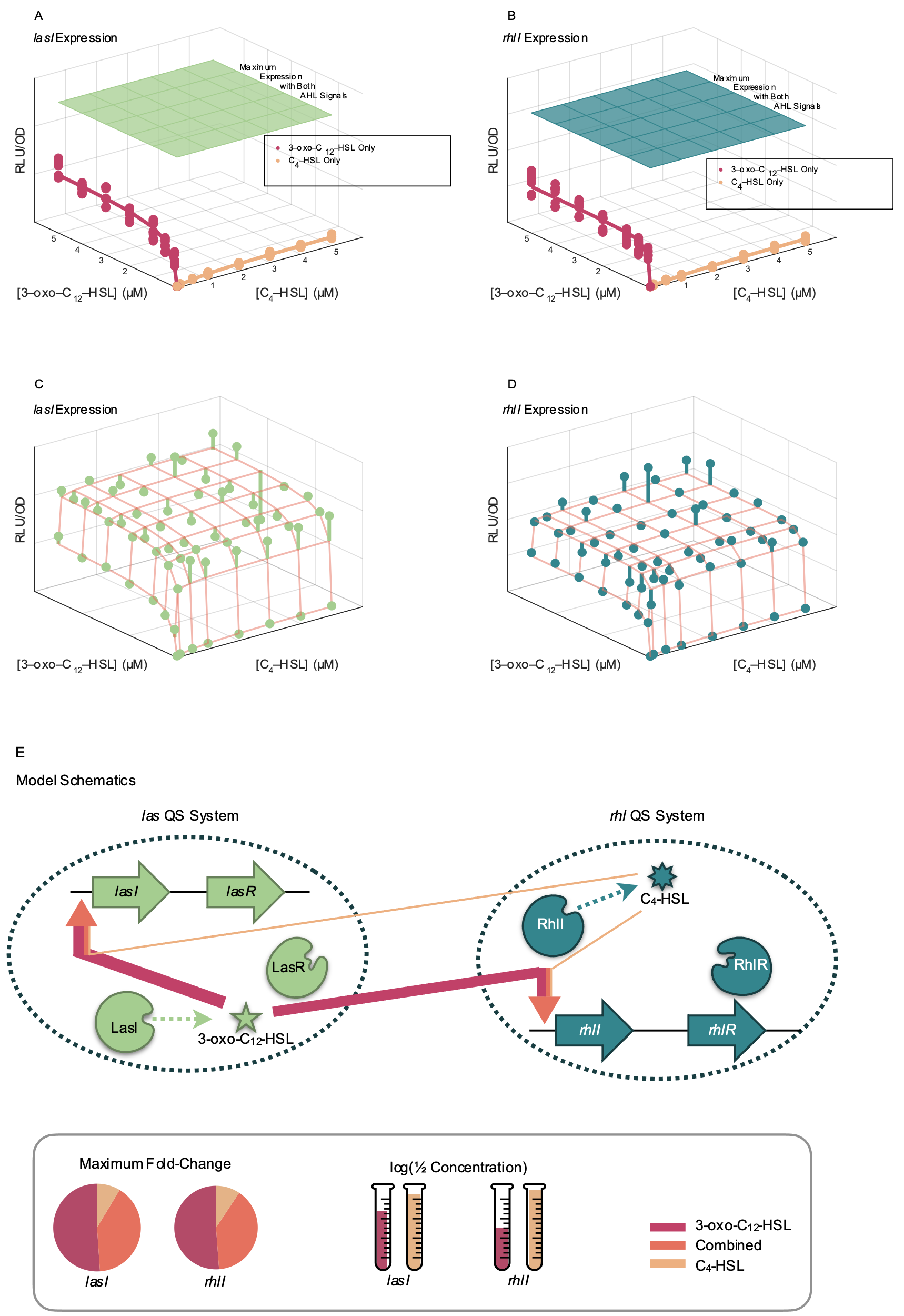
While we find no surprises with 3‑oxo‑C12‑HSL, our experiments with C4‑HSL challenge the conventional hierarchical view. Figure 2C,D shows those results: expression of *las* and *rhlI* increases with higher C4‑HSL concentration. The response of *lasI* (Figure 2C,DA) does not correspond to a simple hierarchy with *las* as the master. Here we find that the *rhl* system affects the *las* system.

To quantify the impact of each signal alone, we model gene expression using Michaelis-Menten kinetics under quasi-steady state assumptions (Methods, Figure 2A-D), allowing us to estimate both the maximal fold-change under signal activation and sensitivity to signal (Table S.3). Our model fits indicate that while the *las* and *rhl* systems have reciprocal impacts, those impacts are not symmetrical. The *las* signal 3‑oxo‑C12‑HSL has a substantially greater influence on gene expression than C4‑HSL. In both cases the potential fold-change from 3‑oxo‑C12‑HSL is approximately six times greater than the potential fold-change from C4‑HSL. Both *lasI* and *rhlI* are also more sensitive to 3‑oxo‑C12‑HSL than to the C4‑HSL as the concentrations required to reach half of maximal expression are roughly 4 times and 30 times higher for the latter.

Figure 2A-D considers the effects of each signal in isolation, but wildtype cells with functioning synthase genes can produce both signals. To understand environments where both signals are present, we use controlled concentrations of both signals in combination. Figure 2E,F presents those results in the form of heat maps. The qualitative responses of both genes are similar: raising the concentration of either signal increases expression regardless of the concentration of the other signal. As with our observations of C4‑HSL alone, these results demonstrate again that the *rhl* system (via C4‑HSL) affects the *las* system (*lasI* expression).

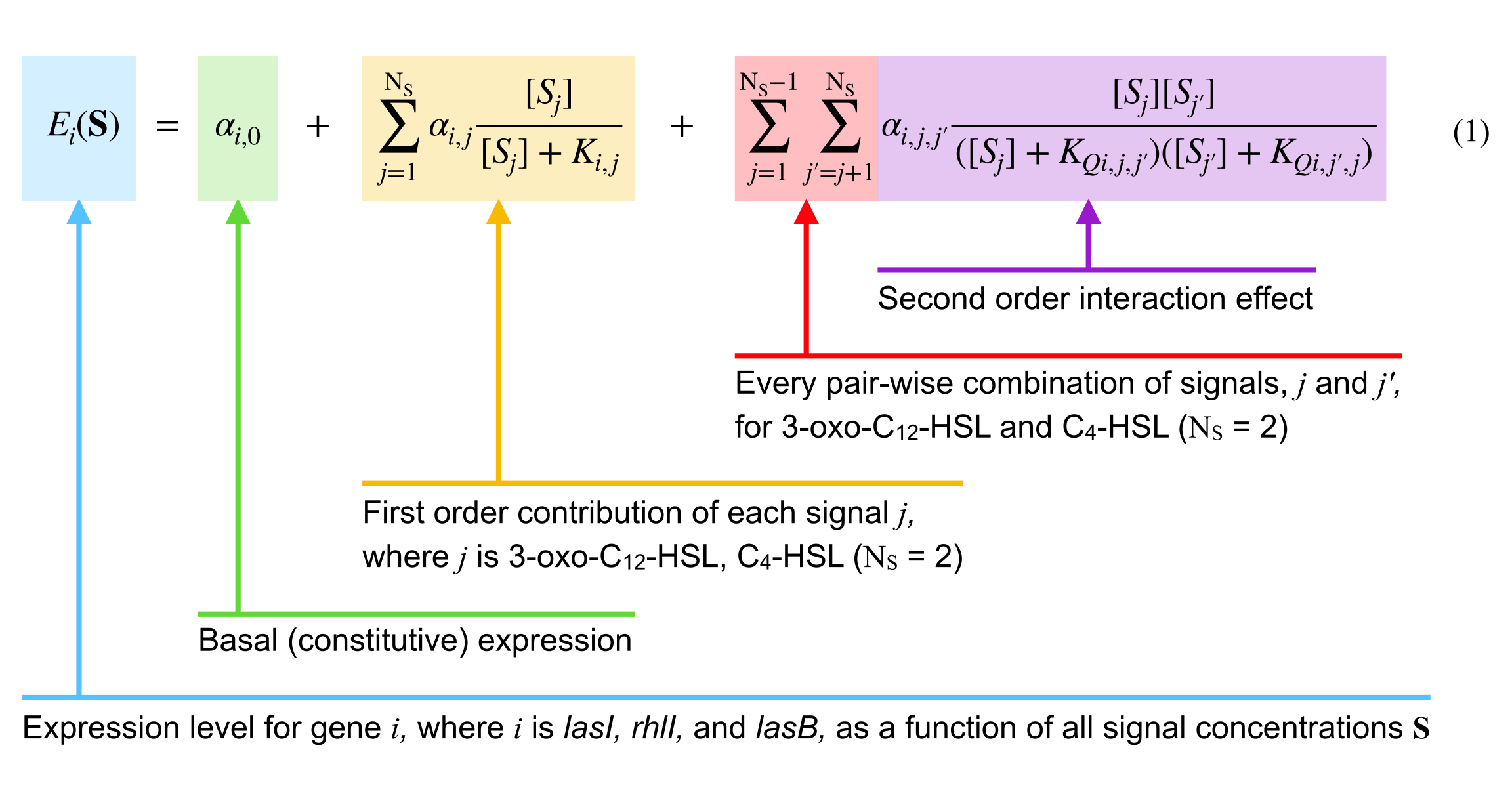
### Quantifying the *las* and *rhl* Interactions Reveals Non-Linear and Synergistic Effects

Having established a simple Michaelis-Menten model for each signal in isolation (Figure 2A-D), we next consider whether that model is sufficient to explain the effect of the signals in combination Figure 2E,F. Can we estimate total expression as the sum of expression induced by each signal alone? Such a response could result from two independent binding sites in the promoter regions (Buchler, Gerland, and Hwa 2003), one site for LasR/3‑oxo‑C12‑HSL and a separate site for RhlR/C4‑HSL. Figure 3A,B clearly shows that we cannot. The maximum expression observed, shown as a “ceiling” in that figure’s panels, far exceeds the sum of the signals’ individual influence. The presence of both signals boosts expression by as much as 30-fold beyond the level of what a simple sum would predict.



**Figure 3. The las and rhl QS systems have a reciprocal, synergistic, and unequal relationship.** (A,B) Neither 3‑oxo‑C12‑HSL nor C4‑HSL alone can achieve maximal expression of lasI or rhlI. Both genes require non-zero concentrations of both signals to achieve maximum expression. The green, flat surfaces in the plots indicate the maximum mean expression level measured across all combinations of signal concentrations. The plotted points represent observed expression levels when C4‑HSL is withheld (red) and when 3‑oxo‑C12‑HSL is withheld (yellow). Lines indicate the model predictions (Equation S.1, parameters in Table S.3). (C,D) Multi-signal models of Equation 1 for lasI and rhlI expression capture the synergistic effects of both signals. Model estimates are shown as orange grid lines. Spheres show the mean value of expression observed at each combination of signal concentrations. Lines extend from these mean values to the relevant grid point for clarity. The coefficient of determination (R2) for the models is 0.82 and 0.77, respectively. Figures S.4 and S.5 present more detailed comparisons between model and observations. (E) Graphical summary of fitted model. Red arrows represent maximum fold-change induction from las’s 3‑oxo‑C12‑HSL and yellow arrows maximum fold-change induction from rhl’s C4‑HSL. The orange component is additional induction from the combination of both signals. Arrow thickness is proportional to fold-change. Inset shows relative contribution of each signal to total maximum fold-change for expression levels of lasI and rhlI, in pie charts and the relative logarithms of half concentration values for each signal as stylized bar charts.

To account for the synergy between the signals, we incorporate a cooperativity term in the gene expression model. Note that the cooperativity term is a multiplication of signals, and it alone cannot explain the full response, as the product is necessarily zero when any signal is absent. This term accounts for any non-additive interaction, for example the ability of one bound transcription factor to recruit the binding of a second transcription factor (Kaplan et al. 2008). Equation 1 shows the result. Each gene has a basal expression level, amplification from each signal alone, and additional amplification from each pair-wise combination of signals. The interaction from these pair-wise combinations captures the cooperative enhancement from the combined signals.



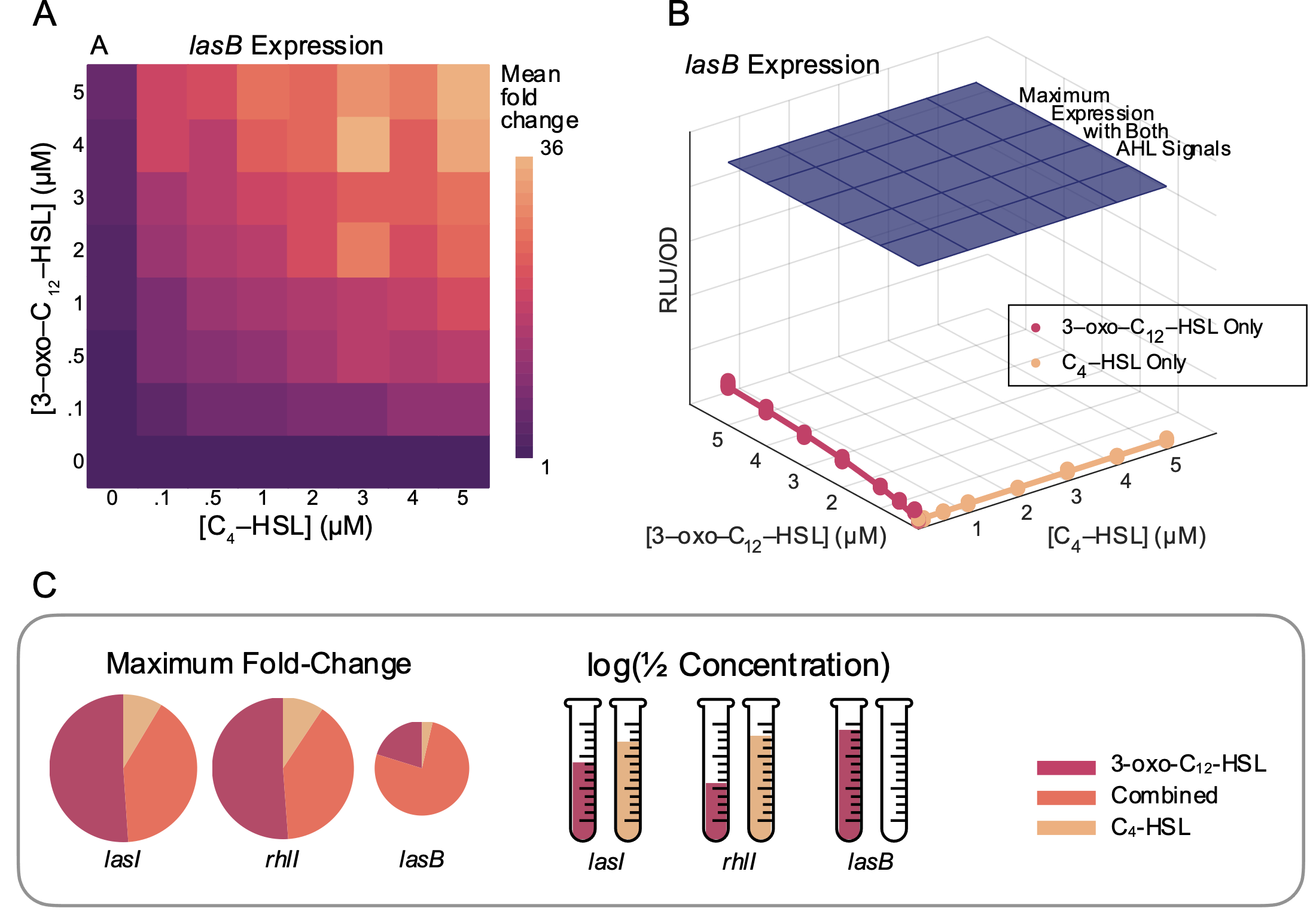
For both *lasI* and *rhlI* we again minimize the sum of squared errors to estimate parameter values (non-linear regression using the Gauss-Newton algorithm). The resulting multi-signal models in Table S.4 have R2 values of 0.82 and 0.77. Figure 3C,D compares the model estimates with observations. For both genes, the model captures the effect of either signal in isolation and both signals in combination.

The parameter estimates in Table S.4 quantify the relative effect of individual and combined signals. For both *lasI* and *rhlI,* a single signal increases expression no more than 38- or 35-fold. Both signals combined, however, increase expression an *additional* 30- or 27-fold. The maximum expression induced by both signals nearly doubles compared to the maximum expression induced by any signal alone. Figure 3E summarizes the model parameters graphically. It answers the question posed in Figure 1D—the *rhl* system does influence the *las* system—and it shows the relative magnitudes of the effects.

### *las* and *rhl* Synergy Also Shapes Control of Quorum Sensing Effector Genes

Our results, summarized in Figure 3, establish that both AHL signals influence the expression levels of both synthase genes in a synergistic manner. We note that the methods outlined in Figures 2–3 for the *lasI* and *rhlI* synthase genes can be applied to any QS-controlled gene of interest and can quantify dual (or more) signal control over expression levels. Here we look at *lasB,* a classic QS effector gene that codes for the secreted digestive enzyme LasB and is widely used as a model of QS-mediated virulence (Casilag et al. 2016; Cigana et al. 2021) and cooperation (Diggle et al. 2007; Allen et al. 2016; Sexton and Schuster 2017).

The prior literature provides a clear expectation that *lasB* expression will be positively influenced by both AHL signal molecules. (Pearson, Pesci, and Iglewski 1997; Nouwens et al. 2003), but does not provide a quantitative summary of the relative importance of each signal’s contribution—alone and via synergistic effects. Using the approach outlined above, we measure luminescence of a *lasB* reporter in an AHL signal null strain exposed to defined, exogenous concentrations of both AHL signals, revealing maximal activation under dual-signal exposure (Figure 4A). Fitting Equation 1 to the data highlights that *lasB* expression is dominated by the synergistic combination of both signals rather than either signal in isolation (Figure 4B). Notably, *lasB* expression is more sensitive to C4‑HSL than to 3‑oxo‑C12‑HSL. (Figure 4C).

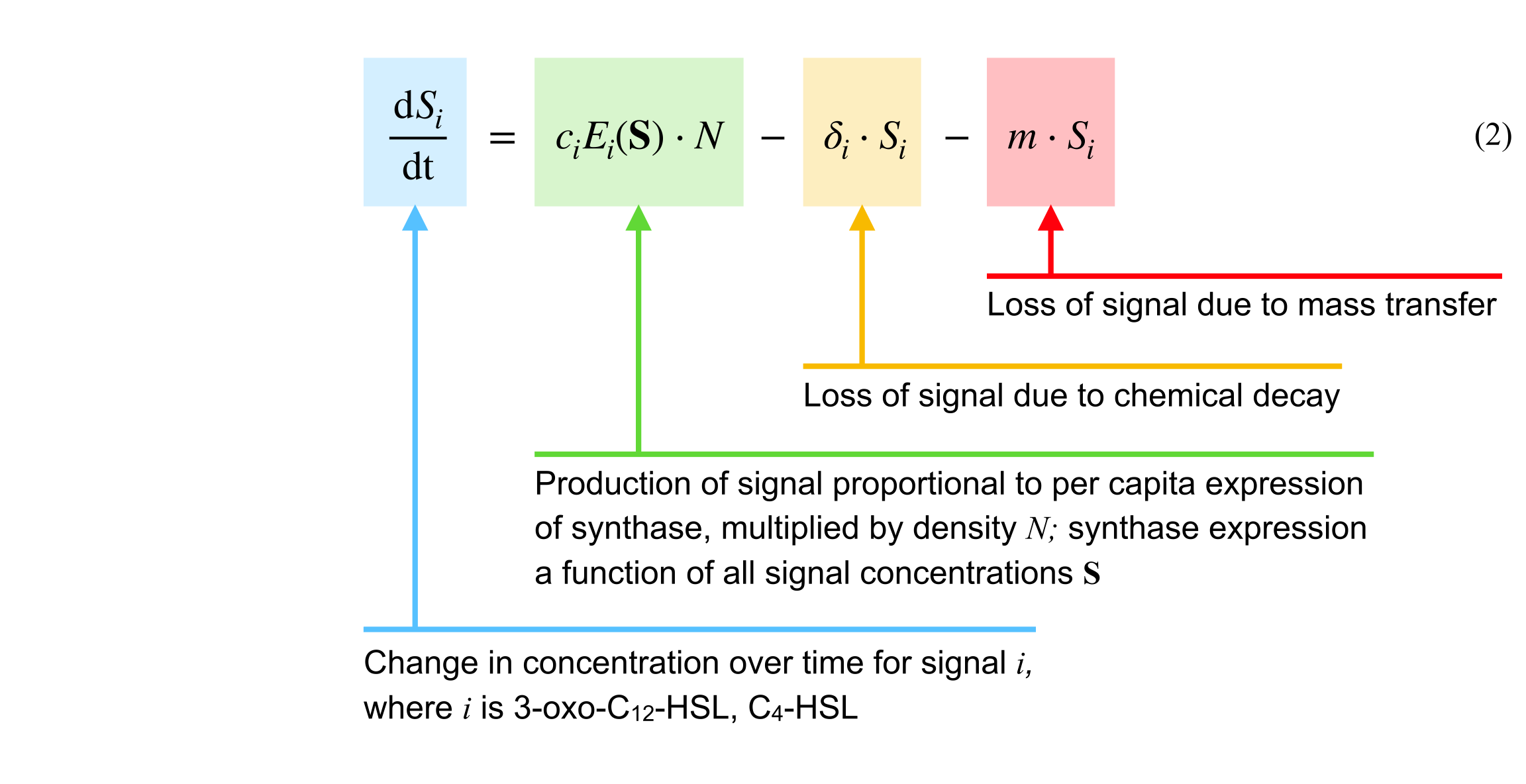


**Figure 4. Expression of lasB is maximal in the presence of both C4‑HSL and 3‑oxo‑C12‑HSL.** (A) Heatmap showing fold-change in RLU/OD values compared to baseline with no exogenous signals. (B) Surface plot showing raw RLU/OD values comparing maximum measured expression (blue “ceiling”) with observed expression levels when C4‑HSL is withheld (red) and when 3‑oxo‑C12‑HSL is withheld (yellow). Lines indicate the model predictions (Equation S.1). (C) Summary of model parameters in context of lasI and rhlI illustrated as in Figure 3. Figure S.3 shows underlying expression data for full time course of experiments; Table S.5 lists the parameter values, and Figures S.6 and S.7 provide a detailed comparison of model predictions and observations.

### Mathematical Models Incorporating Multi-signal Interactions Predict Quorum Sensing Response to Environmental Variation

Our parameterized gene expression model (Equation 1) predicts gene *i*‘s expression *Ei* as a function of the AHL signal environment **S** (Figures 3C,D and S.6), which leaves open the question of how the signal environment **S** relates to underlying dimensions of environmental variation—the hypothesized sensing targets of quorum sensing (population density (Fuqua, Winans, and Greenberg 1994), diffusion (Redfield 2002), efficiency (Hense et al. 2007), containment (Boedicker, Vincent, and Ismagilov 2009), genotypes (Eldar 2011; Allen et al. 2016), combinations (Cornforth et al. 2014), etc.)

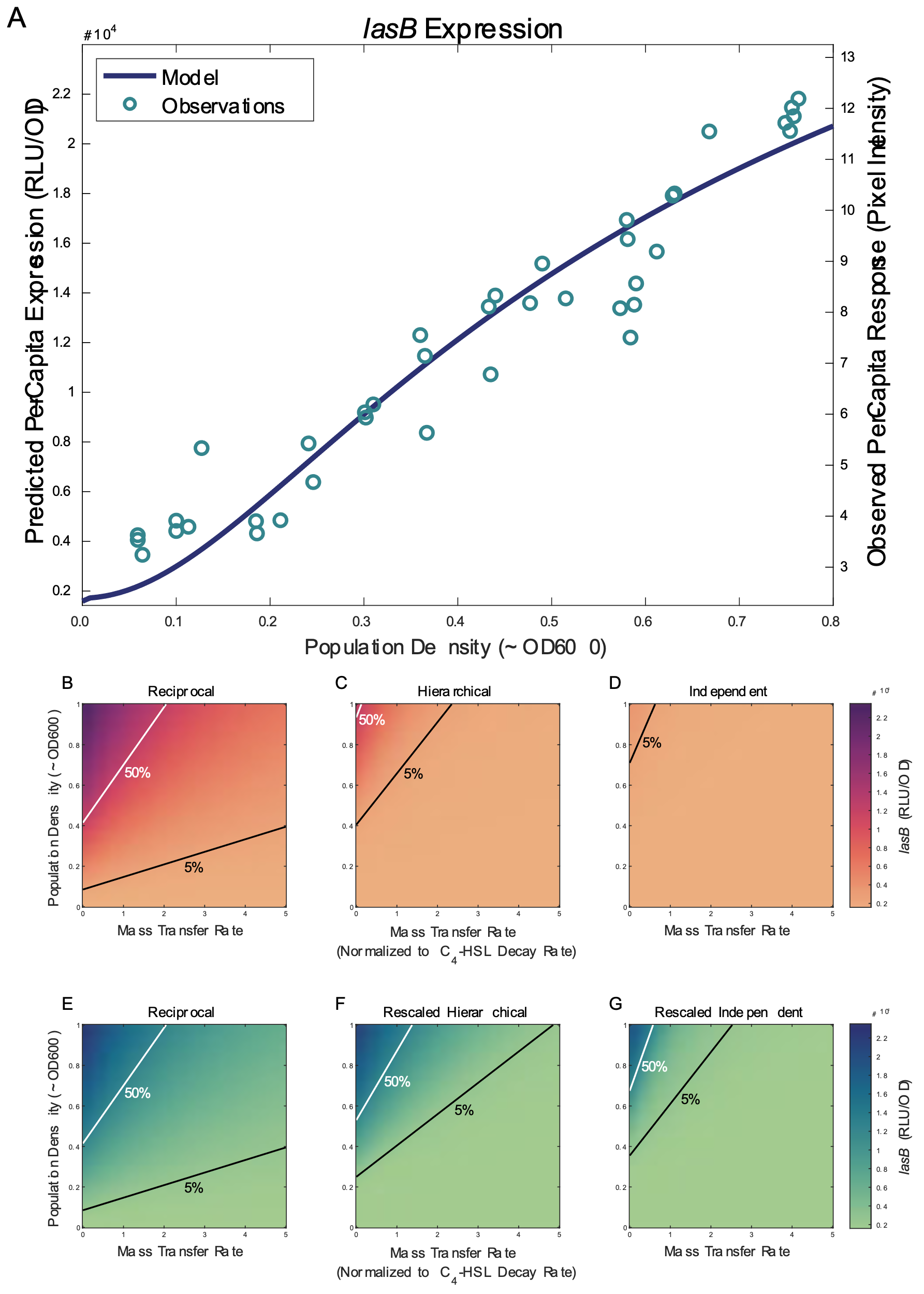
To connect our gene expression model to critical environmental dimensions of bacterial population density and mass transfer (e.g. diffusion or advective flow), we build on previous models of extracellular signal dynamics (James et al. 2000; Dockery and Keener 2001; Ward et al. 2001; Brown 2013; Cornforth et al. 2014). We assume that signal concentration increases in proportion to the corresponding synthase’s expression level, multiplied by the number of cells expressing synthase, and decreases due to a constant rate of decay and removal via mass transfer. These assumptions lead to the differential equation model of Equation 2, where *Si* is the concentration of signal *i*, *Ei* (**S**) is the expression level of the synthase for signal *i* (as a function of both signal concentrations, **S**, see Equation 1) and *ci* a proportionality constant, *N* is the population density; *𝛿i* is the decay rate of signal *i,* and *m* is the rate of mass transfer.



This equation models the dynamics of extracellular signal concentrations in response to environmental conditions defined by density *N* and mass transfer rate *m.* While it is possible to derive analytical equilibrium solutions to Equation 2 for either independent (Figure 2, Equation S.1) or synergistic (Equation 1) signal activation *Ei*(**S**), the resulting solutions are cumbersome and do not yield clear insights into system behavior. (See supporting information.)

To numerically solve for equilibrium signal values in Equation 2 that result from given values of population density and mass transfer rate, we derive parameter estimates for synergistic *Ei*(**S**) from our experimental data (Equation 1, Table S.4). The remaining parameters, *ci* and *𝜹i*, we estimate from published literature as detailed in the supporting information and summarized in Table S.3 and Figure S.8. We can connect these equilibrium signal values to QS-controlled effector behavior using our model of *lasB* expression (Equation 1 as parameterized by Table S.5). Integrating these steps allows us to probe how *lasB* expression varies as the social (*N*) and physical (*m*) environmentals change.

In Figure 5A we illustrate our integrated model prediction of *lasB* expression as a function of change in stationary phase population density, *N* (solid line, Figure 5A), capturing the causal chain from environment *N* to signal levels **S** (Equation 2) and signals **S** to *lasB* expression (Equation 1, Figure 4, Table S.5). To test this prediction using an independent dataset, we use previously published data on *lasB* expression as a function of stationary phase density (Rattray et al. 2022). Our model prediction is in strong agreement with the independently derived data (R2 = 0.91), demonstrating not only the utility of our approach but also providing theoretical support for the conclusions of the prior experimental study (Rattray et al. 2022): QS controlled behaviors in *P. aeruginosa* are not governed by a critical cell density or ‘quorum’ threshold separating off/on states, but are better described as producing graded or rheostatic responses to changes in stationary phase density.



**Figure 5. lasB responds to changes in population density and mass transfer in a graded manner.** (A) Reaction norm (Stearns 1989) showing predicted lasB expression level (solid line), as a function of population carrying capacity. For each density value, equation 2 provides an estimate of equilibrium signal concentrations for 3‑oxo‑C12‑HSL and C4‑HSL. The model of equation 1, parameterized by the estimates of Table S.5, then predicts lasB expression from those concentrations. The figure also shows independent experimental observations of lasB expression as a function of bacterial carrying capacity, manipulated by varying the concentration of limiting carbon (Rattray et al. 2022). Model predictions are in good agreement with independent experimental data (R2 = 0.91). Note that the model parameters are not fitted to the data in this figure. (B-G) Heat maps of lasB expression level as a function of mass transfer m and population density N given three quorum sensing architectures. (B-D) Architectures without rescaling; (E-G) architectures with rescaling to standardize maximum expression levels. The lines on each heat map indicate density and mass transfer values for which lasB expression is constant, either 50% of its maximum value (white) or 5% of its maximum value (black). Expression levels calculated from equation 1 model with parameters from Tables S.5, S.6, S.7 and S.8.

### Multi-Signal Architectures Govern Functional Responses to Environmental Variation

Following an initial validation of our quantitative predictions linking environmental properties to QS-controlled behaviors (Figure 5A), we now turn to analyses of alternate model structures to assess how changes in model architecture translate to changes in the ability to sense and respond to differences in environmental conditions. Specifically, we ask: how do reciprocal versus hierarchical versus independent architectures (Figure 1) influence QS signal and effector responses to variation in bacterial density and mass transfer? To answer that question, we recognize that Equation 1 is general enough to model hierarchical and independent architectures in addition to reciprocal architectures. Those alternatives emerge when specific *ɑ* parameter values are equal to zero.

Starting with our full reciprocal activation model (Table S.4), we can derive a *las*-controlled hierarchical model by setting *ɑ*1,2 and *ɑ*1,1,2 to zero (removing influences of the *rhl* system on the *las* system). To derive an independent model we additionally set *ɑ*2,1 and *ɑ*2,1,2 to zero (removing influences of the *las* system on the *rhl* system). These adjustments (parameters summarized in Table S.6) allow the multi-signal architecture to represent the alternate models Figure 1 depicts, but they also diminish the total weight of QS activation processes. To compensate for this diminished activation we also examine rescaled hierarchical and rescaled independent parameter sets, where the maximal activation weightings are held constant across models (parameters in table S.7).

Using our alternate “counterfactual” models, we examine in Figure 5B-G how equilibrium *lasB* expression changes over a range of population densities *N* and mass transfer rates *m*, for all three architectures. Note that in Figure 5A (reciprocal architecture) with *m =* 0, we recover the graded response to increasing stationary density *N* predicted theoretically and confirmed empirically in Figure 5. Looking across alternate models without rescaling (Figures 5B-D) we see that removing activating processes from *rhl* to *las* (hierarchical model) and also from *las* to *rhl* (independent model) leads unsurprisingly to a general weakening of QS maximal response. In the re-scaled models (Figures 5E-G) we normalize maximal expression, so we can contrast functional responses to changes in density and mass transfer. Comparing these panels we can see that changes in architecture change the functional response to environmental variables. In comparison to the hierarchical or independent models, the reciprocal architecture broadens the environmental parameter space where *lasB* expression is elevated (areas above 5% black line, and above 50% white line), and also reduces the slope of activation contours (angles of black and white lines). The change in contour slope indicates a greater robustness to increasing mass transfer, given a reciprocal activation architecture. In Figure S.8 we assess the temporal activation dynamics of *lasB* under the three alternate architectures, and highlight that even under the rescaled model the reciprocal architecture provides the most rapid activation process (given high density and low mass transfer).

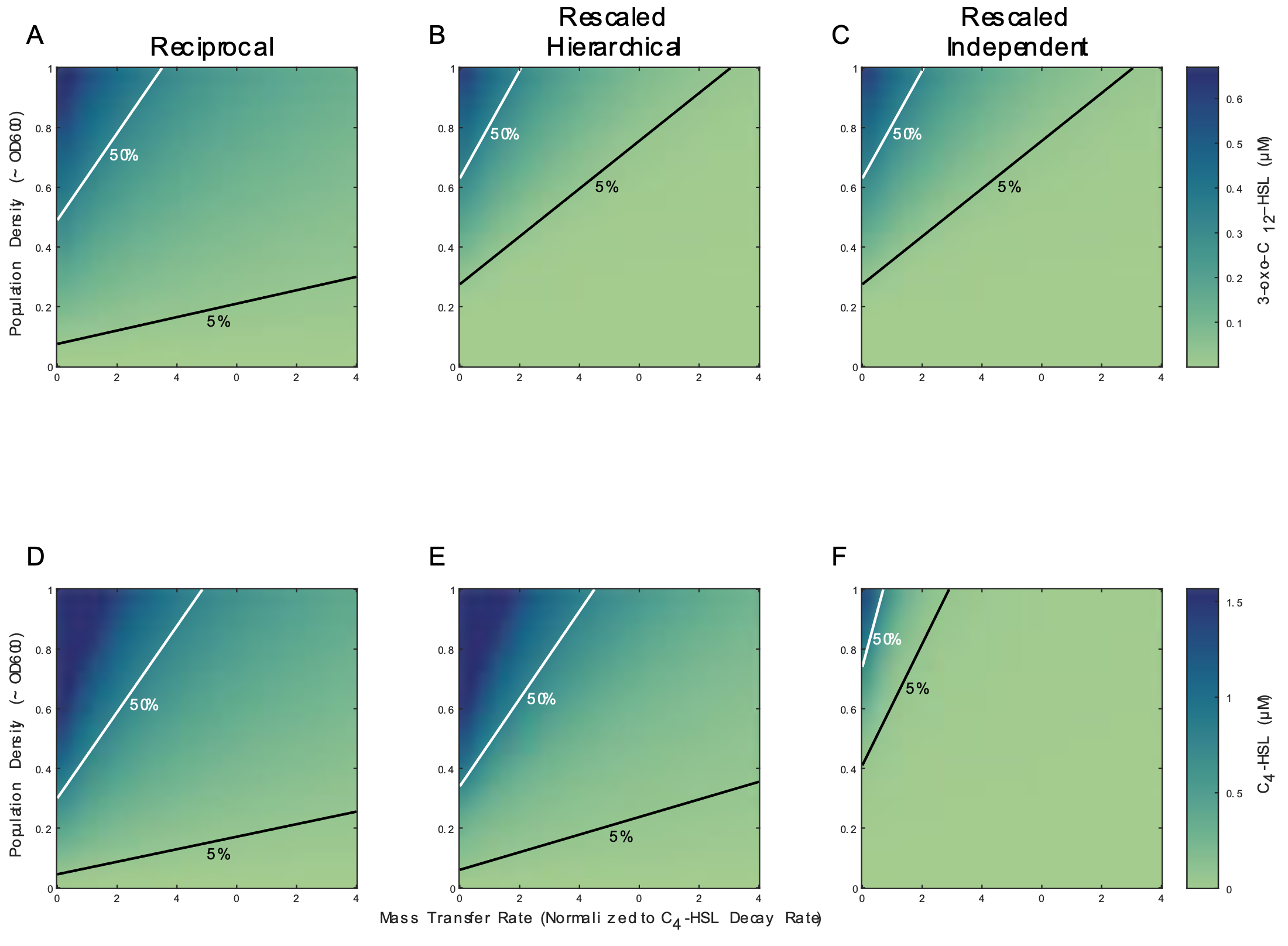
## Discussion

As our knowledge of quorum sensing deepens across species, it is increasingly clear that the use of multiple QS signal molecules is the norm (e.g. *E. coli* (Mayer et al. 2023), *B. subtilis* [Auchtung2006], *P. polymyxa* (Voichek et al. 2020), *V. harveyi* (Henke and Bassler 2004), and *V. cholerae* (Bridges and Bassler 2019)). While this widespread design feature has been probed extensively on a molecular mechanistic scale to produce detailed intracellular “wiring diagrams” (Papenfort and Bassler 2016; Mok, Wingreen, and Bassler 2003). we still lack a general understanding of the dynamical capacities and functional sensing role(s) of these more complex architectures. In this study we use the model QS organism *Pseudomonas aeruginosa* to quantify the dynamical interactions among the *las* and *rhl* signaling sub-systems, and place this dynamical behavior in the functional context of sensing both physical and social environmental variation.

Our experimental data show that the conventional *las-rhl* hierarchical view of QS in *P. aeruginosa* (Figure 1D) is incomplete. Specifically, we quantify a substantial six-fold increase in *lasI* expression compared to basal levels given exposure to the *rhl* signal C4‑HSL (Figure 2C,D). Fitting mathematical models to data, we quantify within- and among-module interactions, and we demonstrate that the *las-rhl* system functions as a biased, reciprocally-activating and cooperative network (Figure 3). To place this intracellular network in an environmental context, we extend our mathematical model to incorporate environmental variation, and find close quantitative support for our model predictions using existing data (Figure 5)A. Finally, we use our parameterized model to examine the impact of changing inter-module wiring, and conclude that the reciprocal architecture results in QS-controlled gene expression that is more sensitive to population density and more robust in the presence of environmental interference (Figure 5B-G).

Turning to the most common functional rationales for QS, the parameterized Figure 5B rejects both the basic “density sensing” and “diffusion sensing” models, as responsiveness to a fixed density is dependent on the level of mass transfer, and vice-versa. While the reciprocal architecture allows greater robustness to increasing mass transfer *m,* it is still the case that maximal *lasB* expression is concentrated in conditions of high density and low mass transfer, consistent with previous models of “efficiency sensing” (Hense et al. 2007) and combinatorial quorum sensing (Cornforth et al. 2014). Under the combinatorial QS model, information on both social and physical conditions (*N* and *m*) are encoded into the distribution of multiple signals (given differences in signal decay, *𝛿i*, and auto-regulation, *ɑi,i*, across signals), which can then be decoded by effectors given non-additive responses to multiple signal inputs. Prior work supports the assumptions of differing rates of environmental decay across AHL signals, and varied non-additive responses across effector genes, including AND-gate control of *lasB* (Cornforth et al. 2014), while our current analysis additionally supports specific predictions on auto-regulation rates (stronger rates of auto-regulation for the more fragile 3‑oxo‑C12‑HSL signal, Table S.4).

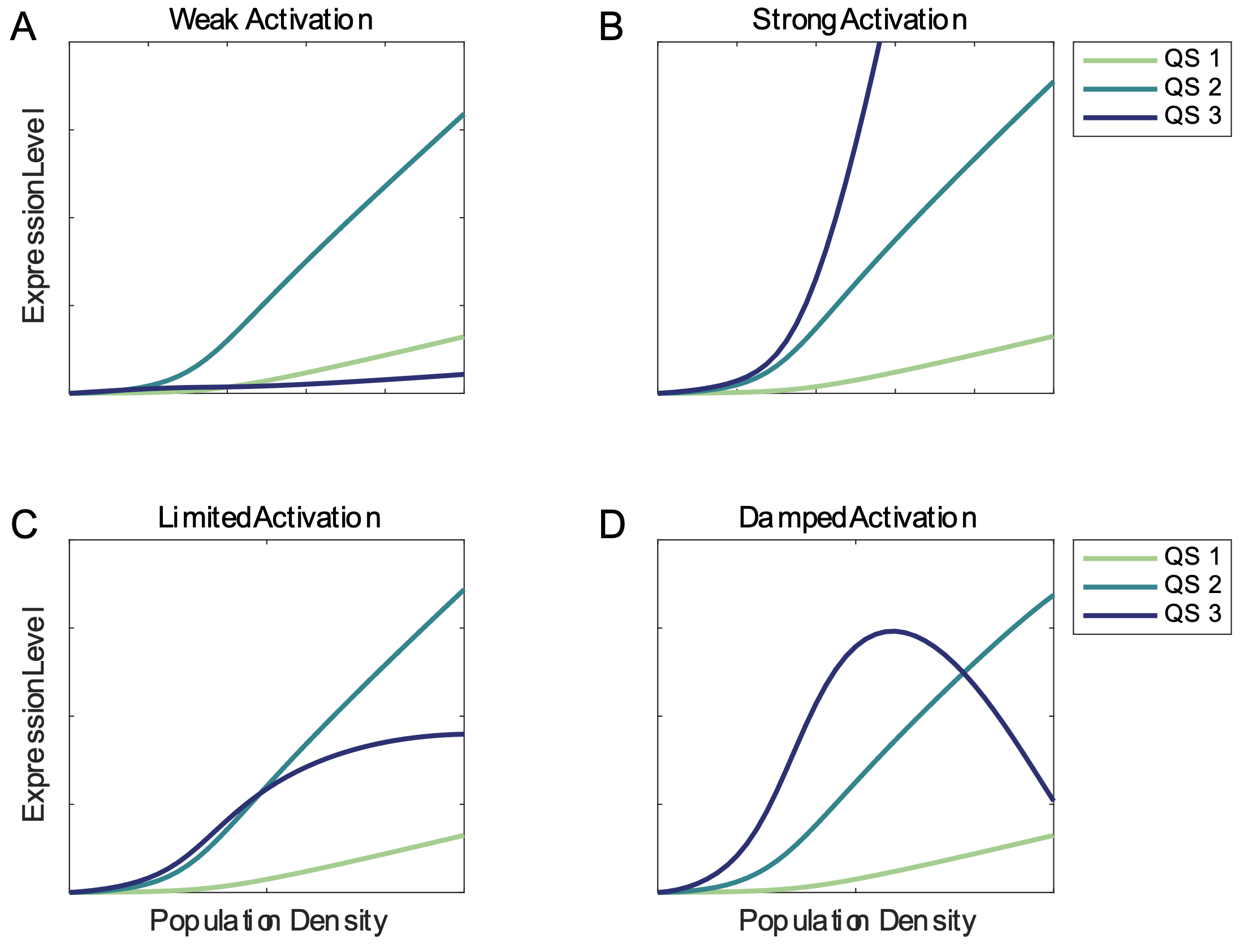
Prior combinatorial QS analyses assumed independent signal activation and a simple threshold activation model for each signal, assumptions that are rejected by the results of the current study. To further assess the combinatorial QS model in light of our new model and data, we plot equilibrium signal concentrations for 3‑oxo‑C12‑HSL and C4‑HSL as a function of environmental conditions, and across our 3 alternate multi-signal architectures (Figure 6). Our analyses illustrate that the prediction of differential signal responses to changing environmental parameters is substantial given the independent architecture (different contour slopes in Figure 6C,F), but is attenuated given the reciprocal achitecture (Figure 6A,D). This attenuation is not surprising given that a reciprocal architecture of activation will strengthen the coupling of activation levels between the two signals, therefore limiting the ability of two signals to report independently on distinct environmental conditions. In Figure S.11 we show that despite the attenuation of differences between the two signals, the ratio of signals still changes substantially (two-fold) across the (*m, N*) parameter space examined, therefore maintaining an encoding of environmental conditions in the distribution of signals.



**Figure 6. Extracellular signal concentration as a function of density and mass transfer varies based on the quorum sensing architecture.** Heat maps of equilibrium 3‑oxo‑C12‑HSL (A-C) and C4‑HSL (D-F) concentration as a function of mass transfer m and population density N for three quorum sensing architectures. Both population density and mass transfer rate are varied over the same ranges for all heatmaps. The lines on each heat map indicate density and mass transfer values for which equilibrium concentration is constant, either 50% of its maximum value (white) or 5% of its maximum value (black). Equilibrium concentrations calculated from equation 1 model with parameters to normalize maximal expression across the three archictures (Tables S.8). Figure S.10 reports the same outputs using non-rescaled model parameters.

By focusing on signal concentration as the factor determining behavior, our approach accommodates multiple possible molecular mechanisms. It does mean, however, that we cannot easily distinguish between alternate mechanistic accounts linking signal exposure to changes in gene expression. For example, C4‑HSL could be causing an increase in *lasI* expression by enabling the formation of LasR dimers, albeit less efficiently than 3‑oxo‑C12‑HSL. Alternatively, it could be the case that the RhlR/C4‑HSL complex serves as an activating transcription factor for *lasI.* Additional experiments would be required to distinguish between these two cases. Our results highlighting gene expression cooperativity (Figures 2E,F and 3A,B) raise additional mechanistic questions. In particular, Figure 3A,B raises challenges for standard additive (Long et al. 2009) or multiplicative (Kaplan et al. 2008) models of gene expression as a function of multiple inputs. Sauer et al. (1995) make related observations for a protein complex in Drosophila melanogaster; both of the developmental regulators BCD and HB alone induce a 6-fold increase by themselves but combine to induce a greater than 65-fold increase. These convergent results across large phylogenetic distances offer a tantalizing possibility that further investigations into the mechanisms of P. aeruginosa quorum sensing interactions can provide insights into more general gene regulatory network models.

Finally, we highlight that our model is not constrained to dual-signal QS, or to mutually enhancing interactions among signal systems. The *pqs* system of *P. aeruginosa,* for example, is both induced by the *las* system and repressed by the *rhl* sytem and it, in turn, can induce the *rhl* system (McGrath, Wade, and Pesci 2004; Lee and Zhang 2015); however, that knowledge alone is not sufficient to predict population behavior. The relative strengths of those interaction can make a qualitative difference in that behavior, as Figure 7 demonstrates. In sum, our methodology provides a general and flexible methodology to quantify the multi-signal “wiring diagrams” of quorum-sensing bacteria and functionally link these wiring diagrams to QS-controlled responses to environmental variation.



**Figure 7. Interaction strength for both induction and repression determines population behavior.** The figure considers hypothetical architectures for a quorum sensing network with three QS systems. The first two, mimicking the architecture of las and rhl, are mutually reinforcing. The third system is both induced and repressed by the other two, matching the reported interactions of pqs. The panels show the synthase expression levels as a function of population density. As the four panels show, even within the constraints of a particular architecture, a wide variety of responses are possible. In panel A, the third system is weakly activated with increasing population density, as indicated by the expression level of the synthase of QS3. Panel B, on the other hand, shows a strong activation of the third system with increasing density. Panels C and D both show a strong initial activation that (C) reaches a limit at which point increases in density does not increase activation or (D) is damped and begins to decline as density increased further. Table S.8 lists the specific parameters used for all panels.

## Methods

### Literature Review

The PubMed database of the US National Institutes of Health was queried on 20 July 2021 using the query [PubMed Search ("review"[Title/Abstract] OR "review"[Publication Type]) AND "quorum sensing"[Title] AND "pseudomonas aeruginosa"[Title/Abstract]](https://pubmed.ncbi.nlm.nih.gov/?term=%28%22review%22%5BTitle%2FAbstract%5D+OR+%22review%22%5BPublication+Type%5D%29+AND+%22quorum+sensing%22%5BTitle%5D+AND+%22pseudomonas+aeruginosa%22%5BTitle%2FAbstract%5D&sort=), resulting in 76 results with publication dates from 1996 to 2021. Papers that incluced a daigram of the gene transcription networks for the *las* and *rhl* quorum sensing systems were further analyzed to interpret the interactions present in those diagrams. Tables S.1 and S.2 show the results. Of the papers analyzed, all show the *las* system positively activating the *rhl* system, and none show the *rhl* system postively activating the *las* system.

### Data Collection

We used three strains for the experimental observations: l*asB:luxCDABE* genomic reporter fusion in NPAO1∆*lasI*∆*rhlI,* *lasI:luxCDABE* genomic reporter fusion in NPAO1∆*lasI*∆*rhlI,* and *rhlI:luxCDABE* genomic reporter fusion in NPAO1∆*lasI*∆*rhlI.* We streaked out all strains in Luria-Bertani (LB) agar at 37°C for 24 hours and then subcultured a single colony in 10 mL LB, incubated at 37°C under shaking conditions (180 rpm) for 24 hours.

We prepared 3‑oxo‑C12‑HSL and C4‑HSL in methanol at 7 different concentrations: 0.1, 0.5, 1, 2, 3, 4 and 5 µM, each diluted from 100 mM stock. We centrifuged all cultures and washed each three times using PBS. We then re-suspended in LB and diluted to an OD (600) of 0.05. We then transferred 200 µl of each culture to a black 96-well plate with a clear bottom and inoculated with signals at the indicated concentrations. We repeated each experiment to generate five replicates. Methanol with no signal was used as a control. The plates were incubated in BioSpa at 37°c for 18 h. Measurements of OD (600) and RLU (Relative Luminescence Units) were collected every hour.

### Data Analysis

We estimated parameter values in Tables S.3, S.4, S.5, and S.6 with non-linear regression by least squares using the Gauss-Newton algorithm (Ratkowsky 1983). Observations were limited to time ranges with peak expression values. (See supporting Information for detailed time course analysis.) Comparisons of model predictions and observed values are available in supporting Information. Equilibrium values shown in Figure 5 were computed using a Trust-Region-Dogleg Algorithm (Powell 1968). Analyses performed and data visualizations created with *MATLAB: Version 9.13 (R2022b)* from The MathWorks Inc., Natick, MA and *Stata Statistical Software: Release 17* from StataCorp LLC, College Station, TX. All original code is available on GitHub at https://github.com/GaTechBrownLab.

Additional third-party modules:

Jann B. “PALETTES: Stata module to provide color palettes, symbol palettes, and line pattern palettes,” *Statistical Software Components* S458444, Boston College Department of Economics, 2017, revised 27 May 2020.

Jann B. “COLRSPACE: Stata module providing a class-based color management system in Mata,” *Statistical Software Components* S458597, Boston College Department of Economics, 2019, revised 06 Jun 2020.

Jann B. “HEATPLOT: Stata module to create heat plots and hexagon plots,” *Statistical Software Components* S458598, Boston College Department of Economics, 2019, revised 13 Oct 2020.

Custom color schemes adapted from seaboarn.

Watson ML. “seaborn: statistical data visualization.” *Journal of Open Source Software* 2021 Apr 06;**6(60)**: doi: 10.21105/joss.03021.

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