High-affinity biomolecular interactions are modulated by low-affinity binders

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Abstract

Molecular interactions play a central role in all biological processes. The strength of these interactions is often characterized by their dissociation constants (K_D). The high affinity interactions ($K_D \leq 10^{-8}$ M) are crucial for the proper execution of cellular processes and are thus extensively investigated. Detailed molecular and biochemical analyses of such high affinity interactions have lent considerable support to the concept of binary on/off switches in different biological contexts. However, such studies have typically discounted the presence of low-affinity binders ($K_D > 10^{-5}$ M) in the cellular environment. In this study, we have assessed the potential influence of such low affinity binders on high affinity interactions. By employing Gillespie stochastic simulations, we demonstrate that the presence of low-affinity binders can indeed alter the kinetics and the steady state of high-affinity interactions. We refer to this effect as 'herd regulation' and have evaluated its possible impact in two different contexts including sex determination in *Drosophila melanogaster* and in signalling systems that employ molecular thresholds. Based on these analyses, we speculate that low-affinity binders may be more prevalent in different biological contexts where the outcomes depend critically on threshold value determinants and could impact their homeostatic regulation.

1 Introduction

Key to all biological processes are biomolecular interactions. Networks of these interactions are organised into pathways, that in many instances, intersect (Huh et al., 2003; Kanehisa et al., 2022). These pathways usually consist of tight binding interactions. In a cellular context, these interactions do not occur in isolation but in the presence of a plethora of other molecules and pathways. This in turn ensures that biomolecules can promiscuously interact with multiple partners with varying degrees of affinity.

In this study, we have analysed the influence of low affinity binders on specific high affinity interactions. The strengths of these interactions are reported in terms of their dissociation constants, K_D. K_D is the equilibrium constant of the reverse reaction given by the ratio of the reverse and forward rate constants. Typically, members of a pathway would interact with their partners, with K_D values in the range of 10^{-6} M to 10^{-14} M (Hu, 2017; Perkins et al., 2010), with lower values corresponding to stronger binding. However, the cellular environment consists of a variety of interactors with variable affinities. In addition to its specific high affinity binding partner(s), a biomolecule could encounter a cohort of other binders with K_D values > 10^{-6} M present in the vicinity. These lower affinity interactions are energetically favourable (negative ΔG) as long as the dissociation constant is < 1M.

Studies of biological processes usually focus only on the important tight binding interactions resulting in productive outcomes. This is in part because these interactions are canonically considered to be central to cellular processes. Moreover, experimental techniques mostly detect strong interactors (Galaway and Wright, 2020; Jiang and Barclay, 2009; Li et al., 2014). Here, we have examined how the kinetics of these high affinity (tight binding) interactions could be altered and/or modulated in the presence of several low affinity binders. For this study, low affinity interactors were conservatively considered to have K_D values of 2 to 5 orders of magnitude less favourable than their stronger counterparts. We also vary the concentration of the low affinity binders to be in the range of 1X to 10X to that of the tight binding partners. This is a conservative estimate as the cellular milieu may contain a higher concentration of the low affinity binding partners, mechanisms for local enrichment of binding partners notwithstanding.

Using these values of binding strengths and relative concentrations, we investigated the effect of weak binding partners on the kinetics and equilibrium of a tight binding interaction. We computed these effects using Gillespie stochastic simulations (Gillespie, 1977, 2013). We found that the presence of low affinity binders transiently alters the kinetics in a manner that could impact non-equilibrium processes. For instance, consider the following interaction.

$$A + B \rightleftharpoons AB$$

Here, the reactants *A* and *B* interact tightly to form the product *AB*. In the environment are several low affinity binders (A_i and B_i), binding to the two reactants. We have studied how the kinetics and changes in steady state at varying levels and binding affinities of A_i and B_i in the cases where – i) there are low affinity binders for either *A* or *B* (B_i and A_i respectively), ii) both reactants have low affinity binders, and iii) there are low affinity binders to *A* and *B* and the A_i's and B_i's are able to interact with each other.

We hypothesize that in a cellular context, such interactions are prevalent as the low affinity binders are likely widespread. Their presence could modulate molecular interactions and hence biological activities. We have modelled the process of sex determination in *Drosophila melanogaster* and signalling thresholds of biomolecular processes in context of these low affinity binders.

Sex-lethal (*Sxl*), a master-switch gene, which determines the female identity, is turned on by the doubling of X chromosome signal in females (Cline and Meyer, 1996). Analysis of mutations in X-chromosome-linked activators has confirmed that activation of early embryonic promoter of *Sxl* (*Sxl-Pe*) is critical in females and thus, underlies the binary fate choice. By contrast, strong repressors capable of affecting sex determination i.e. sex-specific identity and/or viability, have not been identified (Mahadeveraju et al., 2020; Salz and Erickson, 2010). Loss of function mutations in such repressors would result in anomalous activation of *Sxl-Pe* in males, which in turn, can induce male to female transformation or lethality. Here, we hypothesise that such repressors are a set of low affinity binders. A single mutation, typically isolated during genetic screens, would not elicit strong phenotypic consequences (e.g. sex-specific lethality) used as stringent criteria for selection. To examine the possible impact of such repressors, we have modelled this system considering *A* as the switch gene, *B* as the X chromosome signal and A_i and B_i as repressors. We demonstrate that our simulation strategy yields outputs that are consistent with previously reported experimental findings.

Successful activation of biological signalling pathways often requires the signal strength to attain or surpass a defined threshold value. Such systems involve the conversion of a linear change in the signal, to a binary on/off state. Morphogen gradients leading to cellular patterning in a variety of developmental contexts constitute one such example (Wartlick et al., 2009). Although such cases are prevalent in biological systems, precise molecular mechanisms underlying these decisions are not always fully understood. Previous modelling attempts at elucidating the mechanisms have employed models that depend on cellular compartments and/or promiscuous binders (Iyer et al., 2022). In this study, we provide a simple yet broadly applicable mechanism that explains such thresholds using low affinity binders.

2 Results

2.1 The effect of low affinity competitors on strong interactions.

2.1.1 Only one of the strong interactors has competition.

To start with, we examined the effect of low affinity interactors on one of the components of a tight binding complex, i.e., the effect of B_i on the formation of the complex *AB* (see equations E1 and E2). *AB* (dissociation constant $K_D = 10^{-8}$ M) is 3 orders of magnitude stronger than AB_i (dissociation constant $K_{D_i} = 10^{-5}$ M). We have monitored the effect of two parameters, initial levels of the low affinity interactors, B_{i0}, and their binding affinities, K_{D_i}. We analysed the kinetics and steady states of these reactions using the Gillespie stochastic simulation algorithm (Gillespie, 2013).

First, we studied the effect of B_{i0} levels. The simulation was set up with 500 copies of *A* and *B* and with $K_D = 10^{-8}$ M. Kinetics of reactions with different levels of B_{i0}, ranging from 0 to 10000 were analyzed at $K_{D_i} = 10^{-5}$ M. At the beginning of the reaction the entities *A*, *B* and B_i are present as free monomers. Almost instantaneously, all the monomers of *A* are bound to either *B* or B_i, proportional to their relative abundance. The larger the value of B_{i0}, the longer the system takes to equilibrate (figure 1A). This is because the forward rate constants of the formation of *AB* and AB_i are assumed to be equivalent. Consequently, the levels of AB_i may be transiently higher than that of *AB*. AB_i levels decrease while *AB* levels increase since AB_i are more likely to dissociate than *AB.* (Figure 1A). As a result, the average time taken (from 50 replicates) for the formation of *AB* (90% of the maximum possible level, rise time) increases with rising levels of B_{i0} (figure 1E).

Furthermore, we independently examined the effect of variation in the dissociation constants of low affinity interactors, on the formation of *AB.* This was done by varying K_{D_i} from 10^{-4} M to

10^{-6} M while maintaining a B_{i0} level of 5000. Decreasing K_{D_i} (increasing the binding strength of the low affinity binder) decreases the rise time of *AB* (figure 1D). When K_{D_i} is greater than 10^{-4} M, the formation of AB complex breaches the 90% level almost instantaneously. By contrast, at the other extreme, when K_{D_i} is smaller than ~10^{-5} M, the rise time of the AB complex tends to infinity (figure 1F).

To calculate the amount of AB found at equilibrium, we have analysed the steady state levels of the AB complex at 625 individual combinations of 25 B_{i0} levels ranging from 0 to 9600 and 25 K_{D_i} values ranging from 10^{-3} M to 10^{-8} M, each replicated 25 times. We observed that AB is the major product as long as K_{D_i} is greater than 10^{-7} (figure 1B). However, B_{i0} levels start to affect the amount of AB formed beyond 5000 molecules. The variance in the outcome of the reaction also increases with increasing B_{i0} (figure 1C).

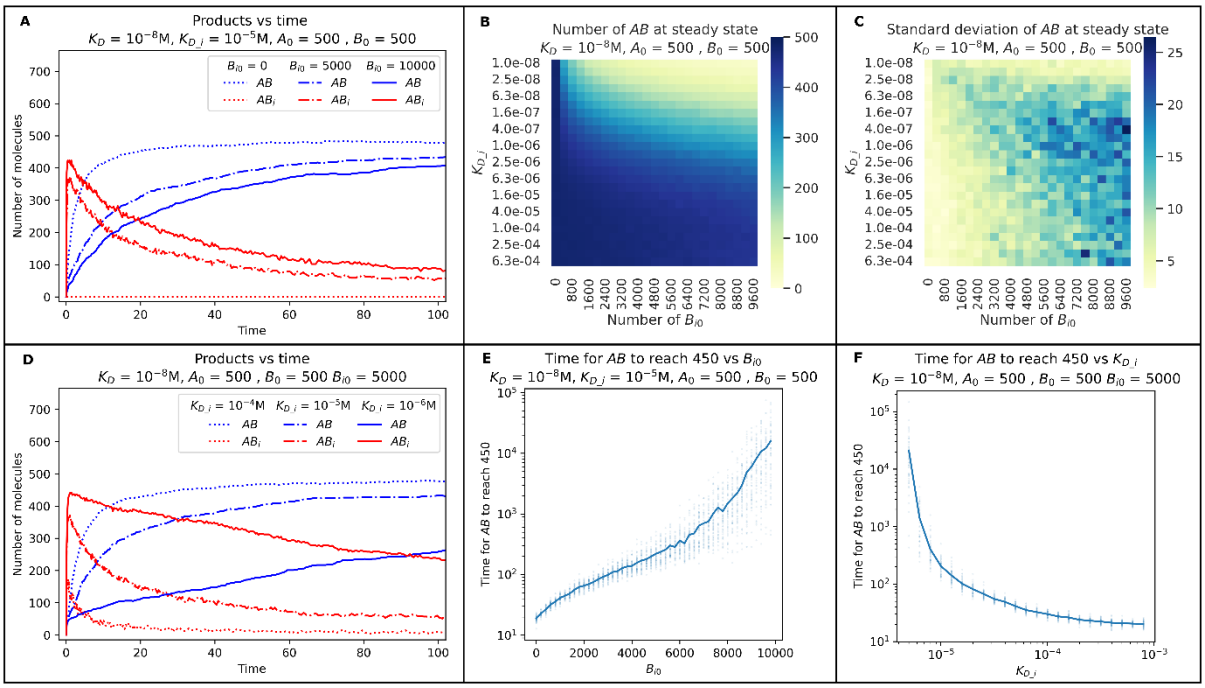

Figure 1: The dependence of complex formation on the starting levels and dissociation constants of the low affinity binder B_i. The kinetics of *AB* (blue lines) and AB_i (red lines) formation at different B_{i0} and K_{D_i} (panels **A** and **D** respectively). The effect of B_{i0} and K_{D_i} on the number of *AB* at steady state (average of 25 replicates per box in panel **B**) with the standard deviations in panel **C**. The time taken for *AB* to reach 450 molecules (90% of the maximum possible value of 500, black dotted line in panels **A** and **D**) at different B_{i0} and K_{D_i} levels (panels **E** and **F** respectively). Panels **A** and **D** show single trajectories taken from triplicate simulations. The lines in panels **E** and **F** are averages over 50 replicates with individual data points shown as scatter plots. The parameters used are shown above the figures.

Since low affinity interactors acting on one component of the high affinity interaction can significantly impact the rate of high affinity interaction, we assessed the effect of low affinity interactors on both the high affinity binders.

2.1.2 Both strong interactors face competition.

In addition to low affinity interactors of *A* (B_i), we also considered the presence of interactors of *B* (A_i) in the reaction. A_i interacts with *B* and forms the complex A_iB (see equations E1, E2 and E3). The dissociation constants of AB_i and A_iB are both described by a single term, K_{D_i}, as we consider them to be equivalent. For this reason, all the results only describe/show AB_i, while the equivalent A_iB is not shown. As in the previous section, the binding affinity of the tight binder (K_D) is 3 orders of magnitude stronger than K_{D_i}. We analyzed the effect of varying

the starting levels of B_i and A_i, as well as their binding affinities K_{D_i} on *AB* formation using Gillespie stochastic simulations.

We first examined the effect of starting levels of B_i and A_i on *AB* formation. Simulations were set up with 500 copies of *A* and *B* and with $K_D = 10^{-8}$ M. The kinetics of reactions with different levels of A_{i0} and B_{i0}, ranging from 0 to 10000 were analyzed at $K_{D_i} = 10^{-5}$ M. We observed that the presence of low affinity binders increases the rise time of *AB* (figure 2A). The time taken for *AB* to equilibrate is an order of magnitude higher than what we noticed when only B_i was present in the system (figures 1A and 2A). Also, the rise time increases exponentially with increasing values of A_{i0} and B_{i0} (figure 2E).

We then investigated the effect of K_{D_i} on *AB* formation at values ranging from 10^{-4} M to 10^{-6} M. Decreasing K_{D_i} increases the time taken for *AB* to equilibrate (figure 2D). Moreover, at $K_{D_i} = 10^{-6}$ M, the reaction seems to prefer the formation of AB_i, the low affinity complex (figure 2D). We also found that the rise time increases with decreasing dissociation constant (figure 2F).

We analysed the steady state of these reactions using 625 different combinations of 25 A_{i0} and B_{i0} levels ranging from 0 to 9600 and 25 K_{D_i} values ranging from 10^{-3} M to 10^{-8} M. AB is not the major product if the number of A_{i0} and B_{i0} are both above 3000 and K_{D_i} is below 10^{-6} M (figure 2B). This is despite a K_D of 10^{-8} M and with 500 copies of both *A* and *B*. The variability in outcome for reactions within this range is also low. Remarkably, the variability in *AB* levels at steady state is comparatively higher betwixt the areas that completely prefer *AB* or AB_i (figure 2C). Thus, the simultaneous presence of the weak binders, A_{i0} and B_{i0}, in the system, has a greater impact on the formation of *AB*, than when only one weak binder is present. This enhancement is sufficient to allow the weaker complex to become the major product. Here, onwards we refer to this effect of weak binders as herd regulation.

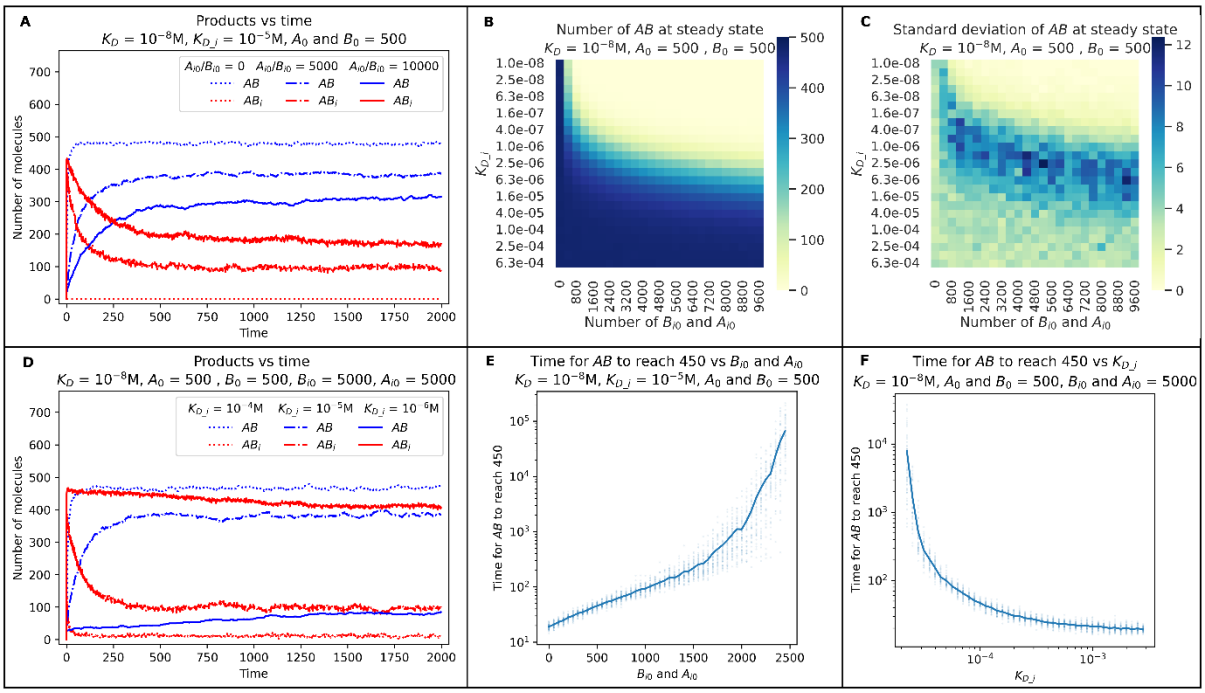

Figure 2: The dependence of complex formation on the starting number and dissociation constant of low affinity binders B_i and A_i. The kinetics of *AB* (blue lines) and AB_i (red lines) formation at different starting levels and dissociation constants of B_i and A_i (panels **A** and **D** respectively). The effect of different starting levels and dissociation constants of B_i and A_i on the *AB* levels at steady state (average of 25 replicates per box in panel **B** and the standard deviations in panel **C**). The time taken for *AB* to

reach 450 (90% of the maximum possible value of 500, black dotted line in panels **A** and **D**) at different starting levels and dissociation constants of B_i and A_i (panels **E** and **F** respectively). Panels **A** and **D** represent single trajectories taken from triplicate simulations. The lines in panels **E** and **F** are averages over 50 replicates. The parameters used are shown above the figures.

We hypothesize that monomeric A_i and B_i present in the system induce the sharp decline in *AB* formation. This is because monomeric *A* and *B* formed by the dissociation of AB_i or A_iB have a higher chance of interacting with A_i and B_i than another tight binding monomer. To validate this idea, we have analyzed the outcome when free A_i and B_i can interact with one another to produce A_iB_i, a system with minimal amount of monomeric A_i and B_i.

2.1.3 Interaction amongst the low affinity competitors decrease herd regulation.

We investigated the effect of low affinity interactors A_i and B_i when they can bind to each other to give rise to the reaction product A_iB_i (E4). The dissociation constant of A_iB_i is denoted by K_{D_cross}. We set the dissociation constant for AB_i and A_iB as 10^{-6} M, a value at which herd regulation favours the low affinity products (figure 2D). We simulated the effect of cross reaction between A_i and B_i on the kinetics of this system using Gillespie stochastic simulations.

We found that decreasing K_{D_cross} (increasing cross reaction strength) increases the rate of *AB* formation (figure 3A). Also, the time taken for *AB* to reach 90% saturation decreases with decreasing K_{D_cross} (figure 3B). Thus, stronger A_iB_i interactions favours more active product formation, thus supporting our hypothesis (see previous section).

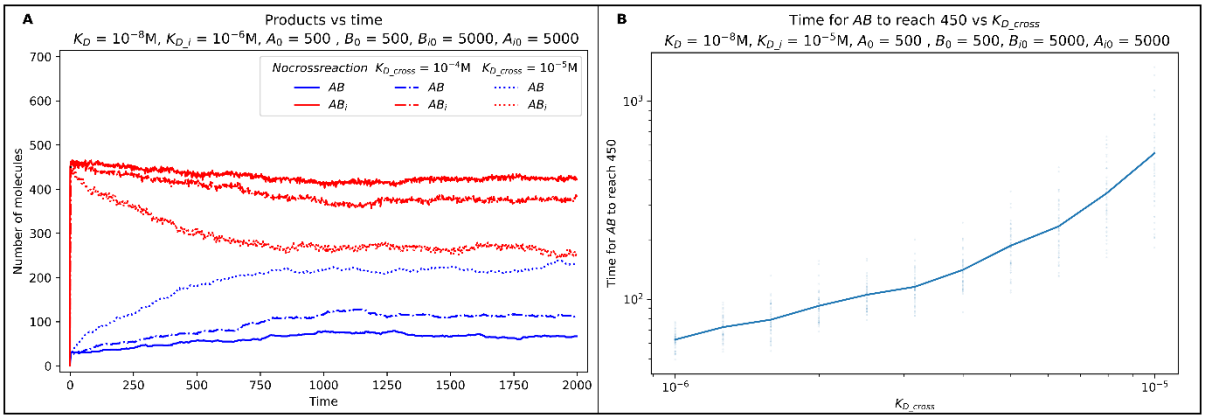

Figure 3: The effect of cross reaction between low affinity inhibitors on herd regulation using Gillespie stochastic simulations. The kinetics of *AB* (blue) and AB_i (Red) complexes at cross-reaction dissociation constants (K_{D_cross}) 10^{-4}M and 10^{-5}M (panel **A**). Panel **B** shows the effect of K_{D_cross} on the time taken for *AB* to reach 450 (90% of the maximum possible value of 500, black dotted line in panel **A**. One of 3 replicates are plotted in panel A. The plot in Panel **B** are averages of 50 replicates (shown as scatter). Starting values of *A*, *B* = 500, $K_D = 10^{-8}$M and $K_{D_i} = 10^{-6}$M. Parameters shown above the figures.

In the last three sections, we have explored the effect of low-affinity interactors on tight binders. In the next two sections, we have examined the effect of herd regulation in deterministic biological contexts and have explored its potential impact on binary outcomes.

2.2 Herd regulation in biological processes.

Here we will illustrate how herd regulation could affect biological processes. In the first case, we will look at sex determination in *Drosophila melanogaster* where a two-fold increase in X chromosome-linked signal generates a binary response during the activation of the *Sxl* gene which determines the female identity. The second example is an illustration of a biological system of receptor and ligand that depend on threshold value determinants.

2.2.1 Example 1: Low affinity binders are responsible for the absence of *Sxl* activation in *Drosophila melanogaster* males

Sexual identity in *Drosophila melanogaster* depends on the female-specific activation of the binary switch gene Sex-lethal (Sxl) before cellularization at cycle 14 (Cline, 1984). Sxl activation, in turn, is regulated by the Sxl establishment promoter *Sxl-Pe*. This process is dependent on X-linked signal elements (XSEs) and autosomal repressors. As females have two X chromosomes, they are able to produce twice the amount of XSEs as compared to males and can specifically activate *Sxl-Pe*. By contrast, *Sxl-Pe* remains off in males who only have one X chromosome. (In insects, the Y chromosome does not contribute to male determination unlike in mammals). The molecular identities of XSEs are well known (Cline, 1988; Cline and Meyer, 1996; Erickson and Cline, 1993, 1991; Kappes et al., 2011; Keyes et al., 1992; Salz and Erickson, 2010). However, the precise mechanism underlying how the doubling of XSE induces the activation of *Sxl-Pe* remains unknown (Deshpande et al., 1995). Of note, no direct repressor that binds to *Sxl-Pe* during early nuclear cycles has been identified, thus far. Moreover, mutations in deadpan (*dpn*) and groucho (*gro*), two known negative regulators of *Sxl-Pe*, display relatively modest phenotypes (Chen and Courey, 2000; Lu et al., 2008; Mahadeveraju et al., 2020; Paroush et al., 1994; Younger-Shepherd et al., 1992).

Here we test the model that multiple low-affinity binders, capable of interfering with the interaction between activators and *Sxl-Pe*, function as regulators of *Sxl*. As the repression depends on several low affinity binders, it is relatively straightforward to explain why the forward genetic screens were unable to identify corresponding mutants. Single mutations typically isolated in the genetic screens will not display the requisite phenotype.

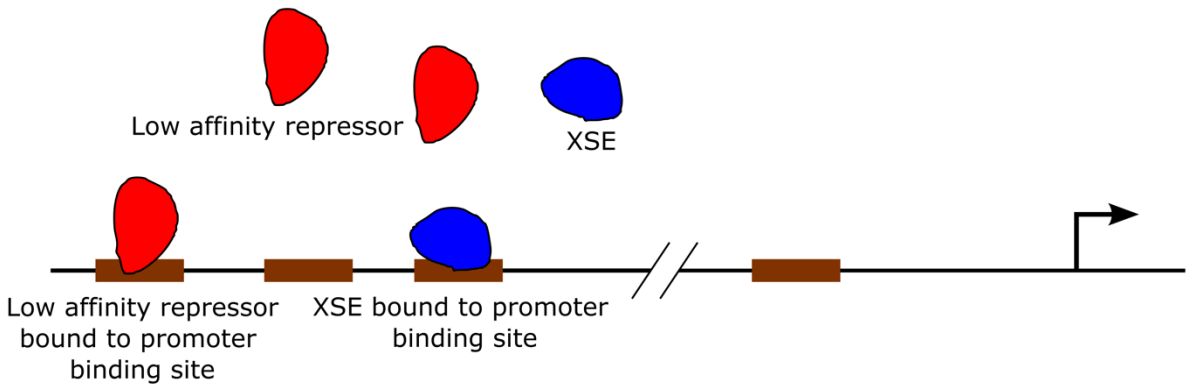

Figure 4: Components of our model of *Sxl-Pe* regulation. The blue objects represent XSEs while the red objects represent the low affinity repressors. The brown boxes represent protein binding sites on Sxl-Pe. The arrow depicts the transcription start site.

Our model posits that sex determination involves one tight binding complex, *AB*, regulated by weaker complexes AB_i (refer to section 2.1.1, equations E1 and E2, and figure 4) . Here, the DNA binding sites in *Sxl-Pe* and XSEs constitute tight binders (*A* and *B*) and form the activating complex (*AB*). Low affinity interactors (B_i) are repressors of *Sxl-Pe* that form the repression complex (AB_i). We assumed that there are 50 binding sites in *Sxl-Pe* (Jinks et al., 2003), 200 XSE activators and 1000 low affinity repressors. The dissociation constant (K_D) of the active complex is 10^{-8} M which is the lower limit of strong binding. The repression complex has a 100 fold lower dissociation constant (K_{D_i}) of 10^{-6} M. Here again, we considered identical forward reaction rates, whereas the reverse reaction rates are determined by the dissociation constant.

Both XSEs and repressors compete for DNA binding sites in *Sxl-Pe*. We consider *Sxl-Pe* to be engaged in active transcription when 90% (45) of the binding sites are occupied by XSEs.

We used Gillespie stochastic simulations to study the kinetics of this system. We calculated the amount of XSEs and repressor bound to *Sxl-Pe* binding sites after 100 units of simulation time at different starting levels of XSEs and repressors. The simulation time scales are relative to the rate constants and may not correspond numerically to biological times.

We first assessed the activity of *Sxl-Pe* at different starting values of XSEs and repressors. As expected, the promoter is predominantly occupied by the repressors when activator levels are low. Increasing XSE levels elevate the number of activating complexes (figure 5A). Subsequently, we examined the effect of changing repressor levels on *Sxl-Pe* activity. Increasing the starting levels of repressors decreases the number of activator-bound *Sxl-Pe* sites. The binding sites are occupied by an equal number of activators and repressors when starting levels of repressors is twice that of starting levels of XSEs. This is despite a 100-fold difference in binding affinities (figure 5B).

Next, we focused on the time taken for the activation of *Sxl-Pe,* both in males and females. In wild-type *Drosophila melanogaster* embryos, activation of Sxl-Pe results in female fate whereas it remains off in males. Furthermore, this is a time-sensitive process as *Sxl-Pe* is active only between nuclear cycles 11-14 and the sex of the embryo is determined by early cellularization. (This is a narrow time window spanning just three nuclear cycles and takes about 30 minutes or ~2000 seconds). For this, we analyzed the ratio of time taken for *Sxl-Pe* activation in male nuclei and female nuclei in *Drosophila* embryos where sex determination occurs autonomously. The calculations accounted for the fact that the XSE levels in females are twice that in males. We found that this ratio is in a narrow range between 2 to 3 even when activator levels are varied between 200-1000 (figure 5C). Thus, activation of *Sxl-Pe* in males takes approximately twice as long as compared to females. We arbitrarily selected a test case where the starting level of XSE was 200 (for males) to study the time dependence of sex determination. We analyzed this over a population of 10,000 male and female simulations, each corresponding to a single nucleus. The time distributions in males and females for *Sxl-Pe* activation show two distinct peaks corresponding to the two sexes, with males (on average) taking appreciably more time for activation than females (figure 5D). Note that Sxl activation occurs over a range of time. Importantly, this pattern of nearly non-overlapping distributions indicates that within a given time interval, *Sxl-Pe* is activated only in females. There is however a small overlap between the male and female distributions (purple region in Figure 5D). This indicates that in a small subset of male nuclei, *Sxl-Pe* is turned on and vice versa in females. If the cellularization event occurs between the two peaks (green dotted line in Figure 5D), our model can temporally distinguish male and female *Sxl-Pe* activation. Thus, our simulations suggest that the time constraint imposed upon the activation of *Sxl-Pe* is partially responsible for its female-specific activation. Furthermore, in the absence of such constraints, Sxl-Pe can be active even in male embryos, which has been demonstrated to lead to detrimental consequences (Cline and Meyer, 1996). Altogether, our analysis recapitulates both the sex-specificity and the temporal dynamics of *Sxl-Pe* activation.

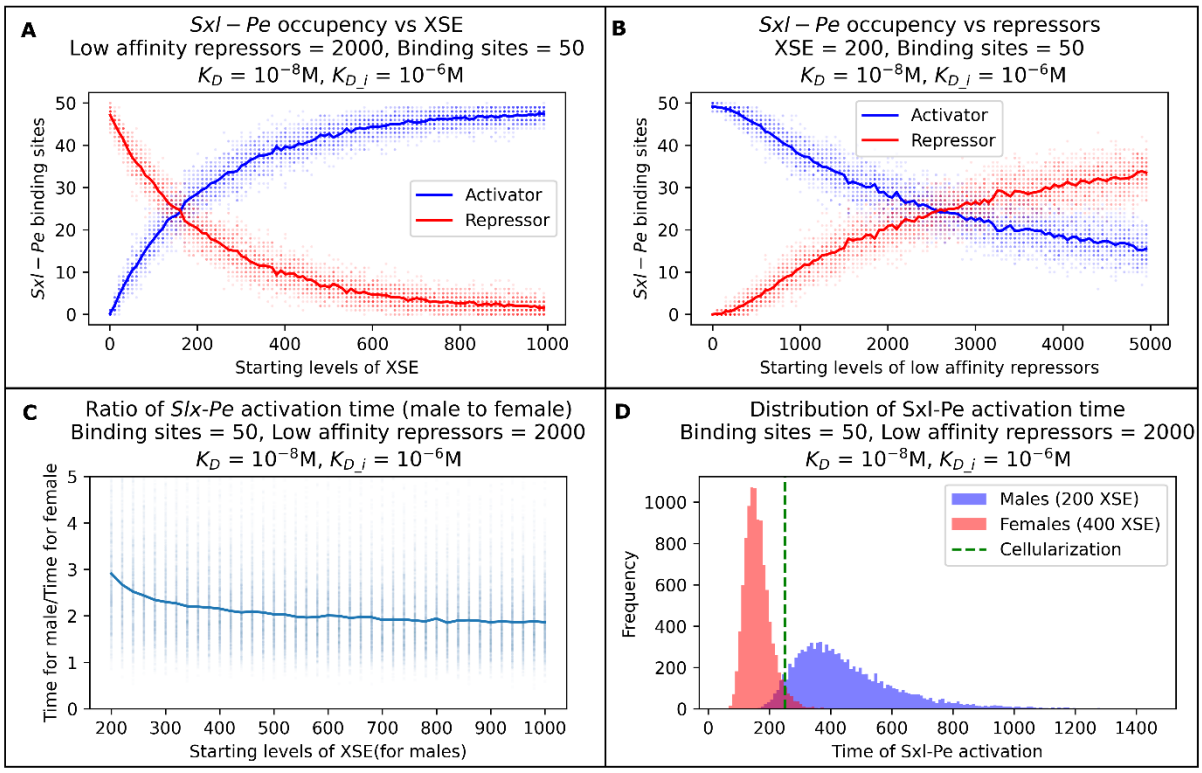

Figure 5: Model of sex determination with one set of low affinity binders. The amount of activating and repressing complexes formed when time = 100 units, at different starting values of XSEs and repressors (Panels **A** and **B** respectively). The ratio of time taken by males vs females to activate *Sxl-Pe* (XSEs occupy 90% of all available *Sxl-Pe* binding sites) (Panel **C**). Panel **D** shows the distribution of time taken for male and female cells to activate across a population of 10000 cells. The simulations were replicated 50 times for each data point in panels **A** and **B** (individual data points shown as scatter of the same colour), and 10000 for panel **C**.

2.2.2 Example 2: Signaling thresholds in biological systems can be explained by herd regulation

In general, molecular thresholds are crucial in several biological signalling events ranging from cell fate determination in early development to neuronal action potentials. Without such thresholds, signals resulting from stochastic noise (inherent to crowded cellular environments) would be able to aberrantly activate (or repress) biological processes. In this section, we describe a general molecular mechanism of how herd regulation can be instrumental in establishing such thresholds in biological systems.

Consider the example where receptor *A* is activated by binding a signalling molecule *B* to form the receptor-signal complex *AB*. The system also contains low affinity binders for the receptor and signalling molecule (B_i and A_i respectively) as we have explored in the previous sections. The receptor-signal complex is assumed to have a dissociation constant of 10^{-8} M whereas both the low affinity complexes have a dissociation constant of 10^{-6} M. We modelled the kinetics of this system using the Gillespie stochastic simulation with 50 receptors and 500 signalling molecules. This is in keeping with the observation that signalling molecules are usually in excess compared to the number of receptors (Cima, 1994). The system also contained 500 copies of each of the two low affinity binders. We monitored the levels of the receptor-signal complex (amount of *AB* formed) after 10000 units of simulation time, starting with different levels of the signal (*B*). The time of 10000 units was chosen to represent a phase of the reaction that is significantly distant from the fast-starting kinetics (see Figure A2C for a

comparison of different time cutoffs). We simulated this system at different starting receptor levels and at levels of the low affinity receptors.

We observed a sigmoidal-like relationship between the number of receptor-signal complexes and the starting levels of the signal. When the number of signaling molecules in the system is below a threshold the rate of increase of the number of receptor-signal complexes is gradual. As soon as the number of signaling molecules exceeds the threshold the number of receptor-signal complexes increases sharply (sigmoid-like). Increasing the levels of the signalling molecule do not alter the threshold but only increases the final levels of the active receptor (figure 6A). Interestingly, we see that the threshold value is regulated by the number of low affinity receptors in the system (figure 6B). Whereas, varying the levels of low affinity signalling molecules does not affect the threshold (figure A2A). Thus, the signal threshold is dependent on the level of low affinity receptors in the system, whereas the amplitude of the signal is determined by the number of receptors.

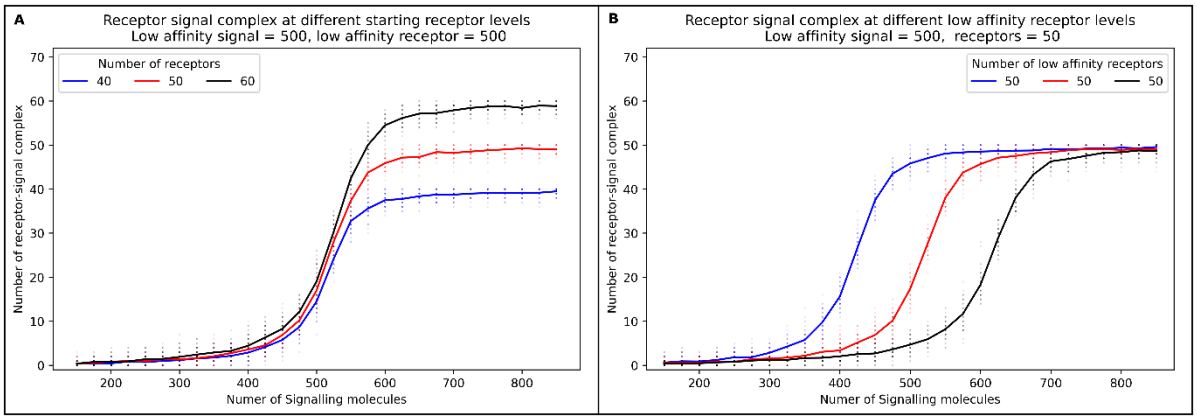

Figure 6: Modelling signalling thresholds in biological systems. All traces (averages of 50 replicates, individual data points shown as scatter) show the relationship between the active receptor and the number of signal molecules and low affinity complexes at different starting levels of the signal. Panel **A** shows this relation at three different starting receptor levels. Panel **B** shows this relation at different starting levels of the low affinity receptor. Plots are averages of 50 replicates and the scatter of the same colours show the individual datapoints.

Iyer et al (2022) analyzed the specification of different cell types under the influence of the Wingless morphogen gradient. They proposed that cellular compartmentalization and receptor promiscuity were critical to elicit a specific response to confer unique cell type identities. Interestingly, this was experimentally verified using the modulators of *Wingless* activity, *Dally* and *Dally like* (B_i) which compete with *Frizzled-2* (*B*) receptor for *Wingless* (*A*) binding. The herd regulation model supports and extends these observations by providing a relatively simple mechanistic explanation for the observed phenomena. We also suggest the presence of undocumented low affinity binders of *Frizzled-2* (A_i). In general, our model could be applied to a number of biological systems where the outcome is contingent upon molecular thresholds.

3 Discussion

Molecular investigations of biological processes almost always focus on interactions that are high affinity and specific. Seldom, if at all, is any importance given to interactions of low affinity/specificity. In part, this could be due to our inability to detect such interactions. In this study, we examine how the presence of low affinity interactors modulates the association of high affinity interactors. We term such modulation of tight binding events by low affinity interactors as ‘herd regulation’.

To illustrate herd regulation, we have considered a simple system of 2 interactors (*A* and *B*) that come together to form a dimeric complex (*AB*). We studied the formation of this complex in the presence of low affinity interactors of both *A* and *B*. The first set of computations considered the formation of *AB* in the presence of B_i, a low affinity interactor of *A*. The K_D of AB_i was 10^3 times weaker than that of *AB*. Despite this disparity in binding strengths, the presence of low affinity competitors affected the kinetics of the formation of *AB*. In fact, the greater the levels of the low affinity interactor the longer it took for the *AB* complex to form. Finally, at steady state, the dominant species is *AB* with nearly no AB_i at $K_{D_i} > 10^{-6}$, regardless of the starting concentrations of B_i.

The kinetics was only further modulated in the background of low affinity interactors to both *A* and *B* (B_i and A_i respectively). In this case, we found that the rise time of the *AB* complex increases with changes in the levels of the low affinity interactors. More importantly, we find situations where *AB* is not the dominant product of the reaction, despite K_D of *AB* several orders of magnitude higher. A_i and B_i when acting along, without the presence of the other, are weak binders and offer weak competition. Only when acting in consort do they become potent competitors. This can be explained by the lack of monomeric *A* and *B* in the system, as all available *A* and *B* are consumed by the formation of *AB*, A_iB and AB_i. This is the case since *A* and *B* contribute to the formation of two complexes each, whereas A_i and B_i are a part of only one complex each. Thus, the products of the dissociation of A_iB or AB_i have a higher probability of encountering A_i or B_i compared to *A* or *B*.

AB is again the dominant product when A_i and B_i can cross react. In this state, there are no monomers present in the system and we see that the effect of herd regulation decreases with the increase in cross reaction strength. Taken together, our results suggest that a 'herd regulated' system can also evolve by mutations that affect the formation of A_iB, AB_i and/or A_iB_i.

In our simulations, we have demonstrated herd regulation with a binary interaction of *A* and *B* giving *AB* with interference from low affinity competitors A_i and B_i. In the crowded cellular environment, reactions could be more complex, involving multiple reactants with varying stoichiometry. It is thus relatively straightforward to imagine that herd regulation would impact such complex interactions more than it does the simple *AB* system. The other important aspect here is that the low affinity competitors could be multiple species. For instance, the interactor A_i could have many homologous molecules in the cell, all of which could collectively constitute the A_i. Given the sizes and abundances of protein families, our estimates of the levels of the low affinity competitors are conservative.

Unlike our simulations, reactions in the cell do not have all the interactors appearing together at the same instant in time. It is conceivable that before one of the strong binding interactors is introduced into the system, the other binding partner and the low affinity interactors are already present. This would only magnify the effects we have discussed above. i.e., the relative rates of reactions could be slower than what we have simulated, and the time taken to equilibrate the system would be longer.

In our computations, we have assumed that the forward rate constants ($k_{forward}$ or k_f) for the high and low affinity interactors are the same. This is not an unusual circumstance in biological processes. For instance, proteins that recognize specific sequences of DNA initially bind non-specifically and diffuse along the DNA to its cognate binding site (Kim and Larson, 2007; Shimamoto, 1999; Wang et al., 2006). Non-specifically binding proteins would also have a similar rate of association with the DNA (Cima, 1994). The rates of dissociation, however, are vastly different and determine the binding affinities. A similar argument can be made regarding

protein-protein (or protein-peptide) and protein-RNA interactions, especially if the binding was predominantly due to complementarity in electrostatics.

We have illustrated herd regulation in biology with two examples. In one, we use protein-protein interactions akin to signal-receptor activation and in another, we use DNA-protein interactions to illustrate sex determination in *Drosophila melanogaster*.

In the example of signaling, we resort to our simple *AB* model (*A* = receptor, *B* = signal or a ligand). The signal (*B*) can bind its cognate receptor *A* or any of the low affinity competitive/homologous receptors A_i. Once *B* is at a level (threshold) where it has saturated all the *A* and A_i's the kinetics is only dependent on the level of B_i (low affinity competing signals). Thus, exhaustion of A_i switches the system, from herd regulation influencing both the tight binders, to one where it affects only a single tight binder. Thus, the threshold is primarily dependent on the amount of A_i, the low affinity receptor, present in the system. This formulation also helps explain earlier observations and computations where the presence of promiscuous receptors (A_i) determines cell fate in *Drosophila melanogaster* development (Iyer et al., 2023).

In the example of sex determination, we analysed a binary switch gene, *Sxl*, whose activity confers female identity. *Sxl* activity is primarily affected by the presence of X-linked signalling elements (XSEs) and autosomal repressors. XSEs are derived from the X chromosome. Thus, females with two X chromosomes activate *Sxl-Pe* while the *Sxl-Pe* of males remains inactive. Sex determination is dependent on the activity of *Sxl-Pe* at the end of the syncytial blastoderm stage of embryogenesis (NC10-13). We have modelled sex determination considering the binding sites in *Sxl-Pe* as *A*, XSEs as *B* and the autosomal repressors as low affinity interactors, B_i. Note that many of the *Sxl-Pe* binding sites are concentrated within 400 base pairs in the promoter. (Estes et al., 1995). We found that the presence of low affinity binders temporally resolves *Sxl-Pe* activation between males and females. XX and XY genotypes would determine female and male states respectively as long as the cellularization occurs at a time point between male and female activation. The primary factor that allows for this temporal decision is the amount of XSEs, the cell cycle at which cellularization happens and the amount of low affinity autosomal repressors present in the system. Among these, the amount of XSEs and timing of cellularization has been experimentally perturbed. Mutations in XSE genes are detrimental to females (Cline and Meyer, 1996). Delayed cellularization generates embryos with more *Sxl-Pe* activation and vice versa (Erickson and Quintero, 2007). It would be interesting to see the effect of perturbing the low affinity binder levels *in vivo*, although this would require the identification of multiple low affinity binders which would need to be mutated simultaneously. Hypothetically, changing the level of the low affinity repressors (herd regulators) could affect the male-female ratio in a population (see figure A3 for distributions of *Sxl-Pe* activation at different low affinity repressor levels).

It will be important to consider these data in a broader, more general context. Even a cursory examination should reveal that binary decisions constitute important cornerstones of biological systems both at the 'micro' and 'macro' levels. It is noteworthy that many of the early foundational discoveries, especially in the field of developmental genetics were aimed at uncovering the presence of a single master determinant that functions as a molecular switch. (Sxl is just one such example of the several well documented 'master switch genes'). As the proper execution of the downstream molecular processes is critically dependent on the early choice, these binary switches have proven to be highly effective means to regulate major outcomes of a variety of determinative events. Notwithstanding the benefits however, one ought to consider a few important caveats that are readily overlooked while considering biological relevance and regulation of binary mechanisms that underlie fate determination.

The genetic and biochemical strategies aimed at the identification and characterization of canonical bio-switches (and their regulatory components) invariably rely on phenotypes that are extreme. For instance, the genetic screens that identified regulators of sex determination relied on either male or female specific lethality. (The nomenclature of the relevant mutations *daughterless*, *sisterless a, b* etc. effectively underscores this feature). As the detection of relevant mutations relied on highly penetrant sex-specific lethality, a number of potentially interesting albeit weak mutations were likely dismissed. Their detection is only possible upon simultaneous deletion or addition of multiple weak affinity factors as approximated in our computational approach.

We have also observed an overlap in the distribution of time taken for male and female *Sxl* activation. This suggests that binary switch systems are not strictly binary. Their outcome is actually two states that in many cases can have small (but not insignificant), overlaps. This becomes especially important in the case of cell autonomous determinative events. For instance, the sex of every single cell in a *Drosophila* embryo is independently determined by the total concentration of X-chromosomal elements. As a result, gynandromorphs consist of cells of both sexual identity and, on rare occasions can survive even up to adulthood. While every instance does not result in a dramatic and demonstrable outcome such as this, it is conceivable that several cells of the 'wrong sex' may be determined in an organism and are eventually eliminated (or rendered ineffective) without any detectable consequence. Analogous scenarios can be envisaged in other biological contexts i.e., a few haltere-like cells could co-exist in wings or some neurons may exhibit partial epithelial traits. Such heterogeneity has been well documented in primordial germ cells in blastoderm stage *Drosophila* embryos while its relevance remains to be determined.

Canonical models suggest that both the initiation and maintenance of the binary switch genes follow a stereotypical path toward a unified outcome. Altogether, while binary decisions may lead to invariable and robust outcomes at a macro- or organismal level, cellular heterogeneity likely exists at a micro- or cellular level. Mechanisms ought to be in place that do not allow such variability to exceed permissible limits leading to detrimental biological consequences. Importantly, the tolerable threshold may differ vastly depending on the cellular or organismal contexts. The mechanistic underpinnings and biological relevance of such mechanisms need closer scrutiny and careful consideration as these represent molecular hurdles that are likely essential components of homeostatic mechanisms.

4 Materials and Methods

4.1 Reactions

Consider the following reaction,

$$A + B \rightleftharpoons AB \tag{E1}$$

Consider the following reactions happening in proximity to E1,

$$A + B_i \rightleftharpoons AB_i \tag{E2}$$

$$A_i + B \rightleftharpoons A_iB \tag{E3}$$

$$A_i + B_i \rightleftharpoons A_iB_i \tag{E4}$$

4.2 Gillespie stochastic simulations

Differential equations can describe the progression of reactions deterministically. But they assume that the reaction volume is infinitely large, and the stochastic behaviour of molecules is cancelled out. This also means that the concentration terms are continuous. But a cell is a small system with relatively few molecules. In such a system the concentration is no longer continuous, and randomness could often play an important role. We use the direct method of the Gillespie stochastic simulation algorithm (Gillespie, 2013, 1977) to simulate such a system. This is a numerical method for simulating the temporal evolution of systems of chemical reactions with randomness. It involves iteratively sampling reaction events and updating the state of the system based on the chosen reaction and the current state of the system. And thus can be used to study the behaviour of complex chemical systems over time, including the effects of noise and uncertainty on the system's dynamics.

We implemented the Gillespie algorithm, in python3. First, the initial state of the system is set, including the number and types of reactants and the reaction rates for each chemical reaction. Then, for each possible reaction, the propensity, a measure of the likelihood that the reaction will occur in the next time step, is calculated based on the reaction rate and the current state of the system. *A* random number generator is used to choose one of the possible reaction events based on the propensities calculated. The state of the system is then updated by adding or subtracting reactants as appropriate. The simulation time is advanced by a time step calculated based on the inverse of the sum of propensity and a random number. The process is repeated until the system reaches a) a pre-specified time b) a specified number of iterations c) when a product crosses a pre-specified level, or d) steady state (described in section 4.2.2).

4.3.1 Parameters used to generate the results

Section 2.1.1: A_0 = 500, B_0 = 500, K_D = 10^{-8} M and C_0 = 10^{-5}M and N_0 = 1000. B_{i0} and K_{D_i} were varied in the section.

Section 2.1.2: A_0 = 500, B_0 = 500, K_D = 10^{-8} M and C_0 = 10^{-5}M and N_0 = 1000. A_{i0}, B_{i0} and K_{D_i} were varied in the section. Note that in all the cases A_{i0} = B_{i0} and their binding affinities were also kept the same and represented by the common term K_{D_i}.

Section 2.1.3: A_0 = 500, B_0 = 500, A_{i0} = 5000, B_{i0} = 5000, K_D = 10^{-8} M, K_{D_i} = 10^{-6} M and C_0 = 10^{-5}M and N_0 = 1000. K_{D_cross} was varied in the analysis.

Section 2.2.1: A_0 = 50, B_0 = 200, B_{i0} = 2000 and K_D = 10^{-8} M, K_{D_i} = 10^{-6} M and C_0 = 10^{-5}M and N_0 = 1000. B_{i0} and B_{i0} were varied in the section.

Section 2.2.2: A_0 = 50, and K_D = 10^{-8} M, K_{D_i} = 10^{-5} M and C_0 = 10^{-3}M and N_0 = 100.. A_0, B_0 and B_{i0} were varied in the section. The simulations were carried out till the simulation time of 10000 units.

Also see section 4.3.3 and appendix 8.3 for the justification for the values provided.

4.3.2 Steady state calculations

For reactions with only B_i present, steady state was assumed to have been reached when the average fluctuation over 1000 steps for every reactant and product is less than 1 (molecule or molecular complex). This was evaluated in intervals of 100 steps.

For reactions with both B_i and A_i, steady state was assumed to have been reached when the average fluctuation of over 1,000,000 steps for every reactant and product is less than 1 (molecule or molecular complex). This was evaluated in intervals of 100,000 steps.

4.3.3 Calculation of propensities

The propensities were calculated using the following equations

$$k_f = 10^5 \text{ M}^{-1}s^{-1}$$

$$k_r = k_f \times K_D$$

$$p_f = \frac{C_0}{N_0} \times k_f$$

$$p_r = k_r$$

Where k_f and k_r are the forward and reverse rate constant respectively. k_f is diffusion controlled and set at 10^5 $M^{-1}s^{-1}$ (Schlosshauer and Baker, 2002).p_f and p_r are the forward and reverse propensities used in the Gillespie stochastic simulations. C_0 and N_0 are used to scale the second order forward rate constant (k_f) based on concentration.

For sections 2.1 and 2.2.1, $C_0 = 10^{-5}M$ and $N_0 = 1000$. For section 2.2.2 $C_0 = 10^{-3}M$ and $N_0 = 100$ (Traces for other values of C_0 can be found in figure A1B) (justifications for the values are provided in appendix section 8.3).

4.4 Data availability

All scripts/programs used in this study along with the data they have produced can be accessed at

http://sites.iiserpune.ac.in/~madhusudhan/Herd_regulation/figures.tar.gz

5 Acknowledgements

We would like to thank - Dr. Neelesh Soni and Aditya Marodia for help with the Gillespie algorithm and debugging scripts, Dr. Chetan Gadgil and Dr. Anirban Hazra for critical reading and criticisms of the manuscript and COSPI lab members. The support and the computational resources provided by the PARAM Brahma facility under the National Supercomputing Mission, Government of India at the Indian Institute of Science Education and Research (IISER) Pune are gratefully acknowledged. SM would like to acknowledge CSIR SRF fellowship. This work was supported by the Department of Biotechnology, Government of India grant to the Indian Institute of Science Education and Research Pune; Department of Biotechnology, India under BICB center grant (BT/PR40262/BTIS/137/38/2022).

6 Competing interests

None

7 References

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8 Appendix

8.1 Effect of changing levels of low affinity signaling molecules on the threshold.

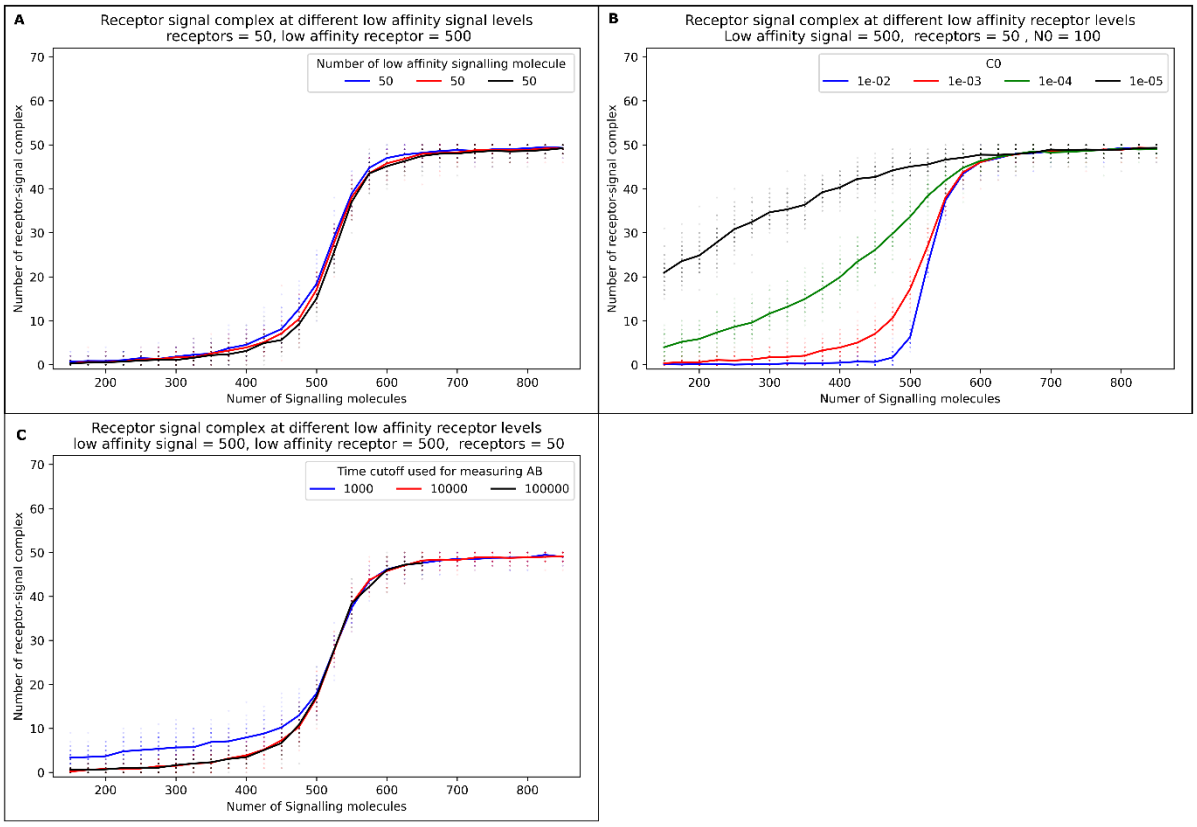

Figure A1: Dependence of receptor activation in varying levels of decoy signaling molecules in panel A, different C_0 values in panel B and different time cutoffs used for sampling in panel C. The parameters used were same as that in Figure 5.

8.2 Male and female Sxl-Pe activation at different starting level of low affinity repressors

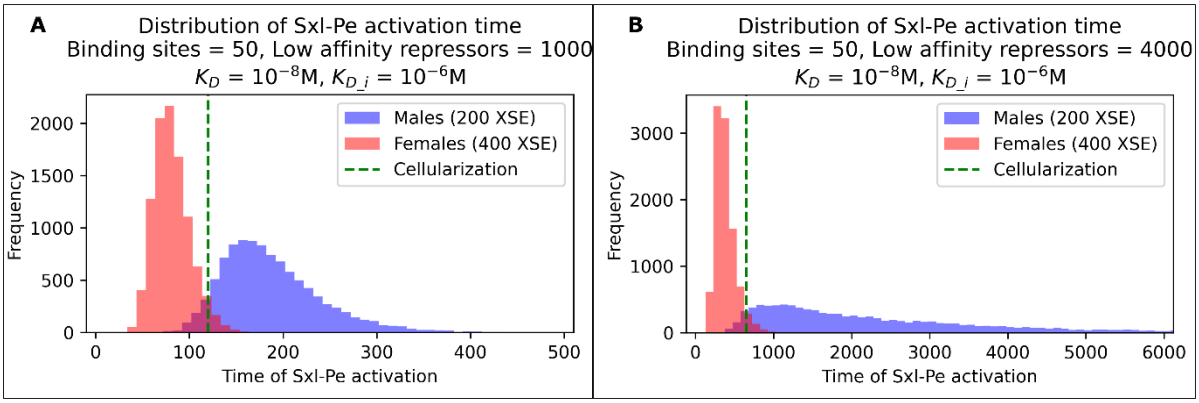

Figure A3: Effect of low affinity repressors on male and female activation times. Panel A shows male and female activation when the starting levels of low affinity repressors are 1000 (half that of figure 5D). Panel B shows the effect of 4000 (double that of figure 5D) low affinity repressors on male and female activation. Parameters are shown in the top of the plots.

8.3 Local concentrations of reactants.

Here we are addressing the physiological relevance of the concentrations of interactors used in the study. To do this, we estimate the volume that contains a single interactor. This in turn gives us the distance that separates two interactors.

The average volume (m^3) occupied by one interactor is calculated from the concentration as

$$Volume = \frac{N_0}{C_0 \times 6.022e23} \times 10^{-3}$$

Where N_0 is the number of interactors and C_0 is the concentration. 6.022e23 is Avogadro's number and 10^{-3} is the conversion factor from dm^3 to m^3.

Assuming that the volume containing the interactor is spherical

$$Volume = \frac{4}{3}\pi r^3$$

Where *r* is the radius of the sphere. The average distance (*d*) that separate two interactors is 2*r*

$$d = 2 \times \sqrt[3]{\frac{3}{4\pi} \times Volume}$$

In this study we have considered reactant concentrations ranging from 10^{-5}M to 10^{-3}M. We have also worked with the number of interactors in the range from (of the order of) 100 – 1000. Substituting these values in equation above gives us interactor distance ranging from 68.2nm to 682 nm. Considering that the sizes of the these interactors is of the order of a few nm and also that cellular and sub-cellular compartments are micrometer scale objects (Maul and Deaven, 1977), the concentrations do not represent overly crowded or unphysical scenarios. In fact, in several instances, the concentrations of cellular interactors are shown to be much higher that our somewhat conservative estimates.

Regulatory processes in biological systems usually occur under conditions of local enrichment, e.g. localization in subcellular compartments (Roth et al., 1989), nuclear speckles (Chen and Belmont, 2019) and lipid rafts in the cell membrane (Simons and Ehehalt, 2002). Such local enrichments have been shown to increase effective concentrations by as much as 5 orders of magnitude (Abrami and van Der Goot, 1999). In comparison, the values of concentrations used in this study are conservative.