

# 1 Co-substrate pools can constrain and regulate pathway fluxes in cell metabolism

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3 Robert West<sup>1</sup>, Hadrien Delattre<sup>1</sup>, Elad Noor<sup>2</sup>, Elisenda Feliu<sup>3,\*</sup>, and Orkun S Soyer<sup>1,\*</sup>

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5 **Affiliations:** <sup>1</sup>School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK.

6 <sup>2</sup>Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot

7 7610001, Israel. <sup>3</sup>Department of Mathematics, University of Copenhagen, Copenhagen,

8 Denmark.

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10 **\*Corresponding Authors:** Orkun S Soyer, University of Warwick, Coventry, CV4 7AL,

11 UK, + 44 (0)24 7657 4251, [o.soyer@warwick.ac.uk](mailto:o.soyer@warwick.ac.uk). Elisenda Feliu, University of

12 Copenhagen, Copenhagen, Universitetsparken 5, 2100, Denmark, +45 (0)35320794,

13 [efeliu@math.ku.dk](mailto:efeliu@math.ku.dk).

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15 **Keywords:** Cell metabolism, metabolic cycles, futile cycles, substrate-induced death,

16 overflow metabolism, metabolic excretions, Warburg effect, Crabtree effect, microbial

17 communities, reaction system dynamics, thermodynamics.

18

19 **Authors contributions:** HD and OSS have devised the study. RW, EN, HD, OSS, and EF

20 performed analyses and simulations, interpreted the results, and wrote the manuscript.

21

22 **Funding key:** This project is funded by the Biotechnology and Biological Sciences Research

23 Council (BBSRC) (grant BB/T010150/1). EF acknowledges funding from the Novo Nordisk

24 Foundation (grant NNF18OC0052483), while OSS acknowledges support from the Gordon

25 and Betty Moore Foundation (grant <https://doi.org/10.37807/GBMF9200>).

26

27 **Acknowledgements:** We would like to thank Wenying Shou for constructive comments on

28 an earlier version of this manuscript, and Dan Davidi for his help with datasets of reaction

29 fluxes and enzyme abundances.

30

## 31 ABSTRACT

32 Cycling of co-substrates, whereby a metabolite is converted among alternate forms via

33 different reactions, is ubiquitous in metabolism. Several cycled co-substrates are well known

34 as energy and electron carriers (e.g. ATP and NAD(P)H), but there are also other metabolites

35 that act as cycled co-substrates in different parts of central metabolism. Here, we develop a

36 mathematical framework to analyse the effect of co-substrate cycling on metabolic flux. In

37 the cases of a single reaction and linear pathways, we find that co-substrate cycling imposes

38 an additional flux limit on a reaction, distinct to the limit imposed by the kinetics of the

39 primary enzyme catalysing that reaction. Using analytical methods, we show that this

40 additional limit is a function of the total pool size and turnover rate of the cycled co-substrate.

41 Expanding from this insight and using simulations, we show that regulation of co-substrate

42 pool size can allow regulation of flux dynamics in branched and coupled pathways. To

43 support these theoretical insights, we analysed existing flux measurements and enzyme

44 levels from the central carbon metabolism and identified several reactions that could be

45 limited by co-substrate cycling. We discuss how the limitations imposed by co-substrate

46 cycling provide experimentally testable hypotheses on specific metabolic phenotypes. We

47 conclude that measuring and controlling co-substrate pools is crucial for understanding and  
48 engineering the dynamics of metabolism.

49

## 50 **INTRODUCTION**

51 Dynamics of cell metabolism directly influences individual and population-level cellular  
52 responses. Examples include metabolic oscillations underpinning the cell cycle (1,2) and  
53 metabolic shifts from respiration to fermentation, observed in cancer phenotypes (3-5) and  
54 cell-to-cell cross-feeding (6-8). Predicting or conceptualising these physiological responses  
55 using dynamical models, however, is difficult due to the large size and high connectivity of  
56 cellular metabolism. Despite this complexity, cellular metabolism might feature simplifying  
57 ‘design principles’ that determine the overall dynamics.

58

59 There is ongoing interest in finding such simplifying principles. Early studies developed a  
60 theory of metabolic pathway structure, concerning the position of ATP generating steps in a  
61 linear pathway, under the assumption of pathway flux optimisation with limited enzyme  
62 production capacity (9). This theory predicted a trade-off between pathway flux and yield  
63 (net ATP generation) (10), which is used to explain the emergence of different metabolic  
64 phenotypes (11). In related studies, several specific models pertaining to enzyme allocation  
65 and optimality have been developed to explain the structure of different metabolic pathways  
66 (12), and the metabolic shifting from respiration to fermentative pathways under increasing  
67 glycolysis rates (8, 13, 14).

68

69 Another conceptual framework emphasized the importance of co-substrate cycling, rather  
70 than net production (e.g. of ATP), as a key to understanding metabolic systems (15). This  
71 framework is linked to the idea of considering the supply and demand structures around  
72 specific metabolites as regulatory blocks within metabolism (16). For example, the total pool  
73 of ATP and its derivatives (the ‘energy charge’) is suggested as a key determinant of  
74 physiological cell states (17). Inspired by these ideas, early theoretical studies have shown  
75 that metabolic systems featuring metabolite cycling together with allosteric regulation can  
76 introduce switch-like and bistable dynamics (18, 19), and that metabolite cycling motifs  
77 introduce total co-substrate level as an additional control element in metabolic control  
78 analysis (20, 21). Specific analyses of ATP cycling in the glycolysis pathway, sometimes  
79 referred to as a ‘turbo-design’, and metabolite cycling with autocatalysis, as seen for example  
80 in glyoxylate cycle, have shown that these features constrain pathway fluxes (22-27). Taken  
81 together, these studies indicate that metabolite cycling, in general, and co-substrate cycling  
82 specifically, could provide a key ‘design feature’ in cell metabolism, imposing certain  
83 constraints or dynamical properties to it.

84

85 Towards better understanding the role of co-substrate cycling in cell metabolism dynamics,  
86 we undertook here an analytical and simulation-based mathematical study together with  
87 analyses of measured fluxes. We created models of enzymatic reaction systems featuring co-  
88 substrate cycling, abstracted from real metabolic systems such as glycolysis, nitrogen-  
89 assimilation, and central carbon metabolism. We found that co-substrate cycling introduces a  
90 fundamental constraint on reaction flux. In the case of single reaction and short linear  
91 pathways, we were able to derive a mathematical expression of the constraint, showing that it  
92 relates to the pool size and turnover rate of the co-substrate. Analysing measured fluxes, we

93 find that several of the co-substrate featuring reactions in central carbon metabolism carry  
94 lower fluxes than expected from the kinetics of their primary enzymes, suggesting that these  
95 reactions might be limited by co-substrate cycling. In addition to its possible constraining  
96 role, we show that co-substrate cycling can also act as a regulatory element, where control of  
97 co-substrate pool size can allow control of flux dynamics across connected or branching  
98 pathways. Together, these findings show that co-substrate cycling can act both as a constraint  
99 and a regulatory element in cellular metabolism. The resulting theory provides testable  
100 hypotheses on how to manipulate metabolic fluxes and cell physiology through the control of  
101 co-substrate pool sizes and turnover dynamics and can be expanded to explain dynamic  
102 measurements of metabolite concentrations in different perturbation experiments.

103

## 104 **RESULTS AND DISCUSSION**

105 **Co-substrate cycling is a ubiquitous motif in metabolism.** Certain metabolites can be  
106 consumed and reproduced via different reactions in the cell, thereby resulting in their  
107 ‘cycling’ (Fig. 1A). This cycling creates interconnections within metabolism, spanning either  
108 multiple reactions in a single, linear pathway, or multiple pathways that are independent or  
109 are branching from common metabolites. For example, in glycolysis, ATP is consumed in  
110 reactions mediated by the enzymes glucose hexokinase and phosphofructokinase, and is  
111 produced by the downstream reactions mediated by phosphoglycerate and pyruvate kinase  
112 (Fig. S1A). In the nitrogen assimilation pathway, the  $\text{NAD}^+$  /  $\text{NADH}$  pair is cycled by the  
113 enzymes glutamine oxoglutarate aminotransferase and glutamate dehydrogenase (Fig. S1B).  
114 Many other cycling motifs can be identified, involving either metabolites from the central  
115 carbon metabolism or metabolites that are usually referred to as co-substrates. Examples for  
116 the latter include  $\text{NADPH}$ ,  $\text{FADH}_2$ ,  $\text{GTP}$ , and Acetyl-CoA and their corresponding alternate  
117 forms, while examples for the former include the tetrahydrofolate (THF) / 5,10-Methylene-  
118 THF and glutamate /  $\alpha$ -oxoglutarate (akg) pairs involved in one-carbon transfer and in amino  
119 acid biosynthesis pathways, respectively (Fig. S1C & D). For some of these metabolites, their  
120 cycling can connect many reactions in the metabolic network. Taking ATP ( $\text{NADH}$ ) as an  
121 example, there are 265 (118) and 833 (601) reactions linked to the cycling of this metabolite  
122 in the genome-scale metabolic models of *Escherichia coli* and human respectively (models  
123 iJO1366 (28) and Recon3d (29)).

124

125 **Cycled co-substrates can act as ‘conserved moieties’ for metabolic flux dynamics.**  
126 Cycling of co-substrate results in their turnover across their different forms e.g.,  $\text{NAD}^+$  and  
127  $\text{NADH}$ . The total pool-size involving all the different forms of a cycled metabolite, however,  
128 can approach a constant value at steady state. In other words, the total concentration of a  
129 cycled metabolite across its different forms at steady state would be given by a constant  
130 defined by the ratio of the influx and outflux rates (see *Supplementary Information (SI)*,  
131 section 2 and 3). In other words, the cycled metabolite would become a ‘conserved moiety’  
132 for the rest of the metabolic system and can have a constant ‘pool size’. Supporting this,  
133 temporal measurement of specific co-substrate pool sizes shows that ATP and GTP pools are  
134 constant under stable metabolic conditions, but can rapidly change in response to external  
135 perturbations, possibly through inter-conversions among pools rather than through  
136 biosynthesis (30).

137

138 **Co-substrate cycling introduces a limitation on reaction flux.** To explore the effect of co-  
139 substrate cycling on pathway fluxes, we first consider a didactic case of a single reaction.  
140 This reaction converts an arbitrary metabolite  $M_0$  to  $M_I$  and involves co-substrate cycling  
141 (Fig. 1A). For co-substrate cycling, we consider additional ‘background’ enzymatic reactions  
142 that are independent of  $M_0$  and can also convert the co-substrate (denoted  $E_A$  on Fig. 1A). We  
143 use either irreversible or reversible enzyme dynamics to build an ordinary differential  
144 equation (ODE) kinetic model for this reaction system and solve for its steady states  
145 analytically (see *Methods* and *SI*, section 3). In the case of using irreversible enzyme kinetics,  
146 we obtain that the steady state concentration of the two metabolites,  $M_0$  and  $M_I$  (denoted as  
147  $m_0$  and  $m_I$ ) are given by:

$$148 \quad m_0 = \frac{k_{in} \cdot K_{m,E_0} \cdot \alpha}{(V_{max,E_0} - k_{in}) \cdot (V_{max,E_a} \cdot A_{tot} - k_{in} \cdot (K_{m,E_a} + A_{tot}))}, \quad m_I = \frac{k_{in}}{k_{out}} \quad (\text{Eq. 1})$$

150 where  $k_{in}$  and  $k_{out}$  denote the rate of in-flux of  $M_0$ , and out-flux of  $M_I$ , either in-and-out of the  
151 cell or from other pathways, and  $A_{tot}$  denotes the total pool size of the cycled metabolite (with  
152 the different forms of the cycled metabolite indicated as  $A_0$  and  $A_I$  in Fig. 1A). The term  $\alpha$  is  
153 a positive expression comprising  $A_{tot}$ , and the kinetic parameters of the enzymes in the model  
154 (see *SI*). The parameters  $V_{max,E_a}$  and  $V_{max,E_0}$  are the maximal rates (i.e.  $V_{max} = k_{cat} \cdot E_{tot}$ ) for the  
155 enzymes catalysing the conversion of  $A_0$  and  $M_0$  into  $A_I$  and  $M_I$  (enzyme  $E_0$ ), and the  
156 turnover of  $A_I$  into  $A_0$  (enzyme  $E_a$ ), respectively, while the parameters  $K_{m,E_a}$  and  $K_{m,E_0}$  are the  
157 individual or combined Michaelis-Menten coefficients for these enzymes’ substrates (i.e. for  
158  $A_0$  and  $M_0$  and  $A_I$ , respectively). The steady states for the model with all enzymatic  
159 conversions being reversible, and for a model with degradation and synthesis of  $A_0$  and  $A_I$ ,  
160 are given in the *SI*. The steady state solutions of these alternative models are structurally akin  
161 to Eq. 1, and do not alter the qualitative conclusions we make in what follows.

162 A key property of Eq. 1 is that it contains terms in the denominator that involve a  
163 subtraction. The presence of these terms introduces a limit on the parameter values for the  
164 system to attain a positive steady state. Specifically, we obtain the following conditions for  
165 positive steady states to exist:

$$166 \quad k_{in} < V_{max,E_0} \quad \text{and} \quad k_{in} < A_{tot} \cdot V_{max,E_a} / (K_{m,E_a} + A_{tot}) \quad (\text{Eq. 2})$$

167  
168 Additionally, the ‘shape’ of Eq. 1 indicates a ‘threshold effect’ on the steady state value of  
169  $m_0$ , where it would rise towards infinity as  $k_{in}$  increases towards the lower among the limits  
170 given in Eq. 2 (see Fig. 1B).

171 Why does Eq. 1 show this specific form, leading to these limits? We find that this is a  
172 direct consequence of the steady state condition, where metabolite production and  
173 consumption rates need to be the same at steady state. In the case of co-substrate cycling, the  
174 production rate of  $M_0$  is given by  $k_{in}$ , while its consumption rate is a function of the  
175 concentration of  $A_0$  and the  $V_{max,E_0}$ . The concentration of  $A_0$  is determined by its re-generation  
176 rate (which is a function of  $K_{m,E_a}$  and  $V_{max,E_a}$ ) and the pool size ( $A_{tot}$ ). This explains the  
177 inequalities given in Eq. 2 and shows that a cycled co-substrate, when acting as a conserved  
178 moiety, creates the same type of limitation (mathematically speaking) on the flux of a  
179 reaction it is involved in, as that imposed by the enzyme catalysing that reaction ( $E_0$  in this  
180 example) (see Fig. 1C&D). We also show that considering the system shown in Fig. 1A as an  
181  
182

183 enzymatic reaction without co-substrate cycling leads to only the constraint  $k_{in} < V_{max,E0}$ ,  
184 while when considering it as a non-enzymatic reaction with co-substrate cycling only, the  
185 constraint  $k_{in} < A_{tot} \cdot V_{max,Ea} / (K_{m,Ea} + A_{tot})$  becomes the sole limitation on the system (see *SI*,  
186 section 3). In other words, the two limitations act independently.

187 To conclude this section, we re-iterate its main result. The flux of a reaction involving  
188 co-substrate cycling is limited either by the kinetics of the primary enzyme mediating that  
189 reaction, or by the turnover rate of the co-substrate. The latter is determined by the co-  
190 substrate pool size and the kinetics of the enzyme(s) mediating its turnover.

191

192 **Co-substrate cycling causes a flux limit on linear metabolic pathways.** We next  
193 considered a generalised, linear pathway model with  $n+1$  metabolites and arbitrary locations  
194 of reactions for co-substrate cycling, for example as seen in upper glycolysis (Fig. S1). In this  
195 model, we only consider intra-pathway metabolite cycling, i.e. the co-substrate is consumed  
196 and re-generated solely by the reactions of the pathway. Here, we show results for this model  
197 with 5 metabolites as an illustration (Fig. 2A), while the general case is presented in the *SI*  
198 section 4.

199 We find the same kind of threshold dynamics as in the single reaction case. When  $k_{in}$   
200 is above a threshold value, the metabolite  $M_0$  accumulates towards infinity and the system  
201 does not have a steady state (Fig. 2B). A numerical analysis, as well as our analytical  
202 solution, reveals that the accumulation of metabolites applies to all metabolites upstream of  
203 the first reaction with co-substrate cycling (Fig. 2C and *SI* section 4). Additionally,  
204 metabolites downstream of the cycling reaction accumulate to a steady state level that does  
205 not depend on  $k_{in}$  (Fig. 2C and Fig. S2). In other words, pathway output cannot be increased  
206 further by increasing  $k_{in}$  beyond the threshold. Finally, as  $k_{in}$  increases, the cycled metabolite  
207 pool shifts towards one form and the ratio of the two forms approaches zero (Fig. 2C).

208 An analytical expression for the threshold for  $k_{in}$ , like shown in Eq. 2, could not be  
209 derived for linear pathways with  $n > 3$ , but our analytical study indicates that (i) the threshold  
210 is always linked to  $A_{tot}$  and enzyme kinetic parameters, and (ii) the concentration of all  
211 metabolites upstream (downstream) to the reaction coupled to metabolite cycling will  
212 accumulate towards infinity (a fixed value) as  $k_{in}$  approaches the threshold (see *SI* section 4).  
213 In Figure 2, we illustrate these dynamics with simulations for a system with  $n=4$ .

214 We also considered several variants of this generalised linear pathway model,  
215 corresponding to biologically relevant cases as shown in Fig. S1. These included (i) intra-  
216 pathway cycling of two different metabolites, as seen with ATP and NADH in combined  
217 upper glycolysis and fermentation pathways (Fig. S3, *SI* section 5), (ii) different  
218 stoichiometries for consumption and re-generation reactions of the cycled metabolite, as seen  
219 in upper glycolysis (Fig. S4, *SI* section 6), and (iii) cycling of one metabolite interlinked with  
220 that of another, as seen in nitrogen assimilation (Fig. S5, *SI* section 7). The results in the *SI*  
221 confirm that all these cases display similar threshold dynamics, where the threshold point is a  
222 function of the co-substrate pool size and the enzyme kinetics.

223

224 **Cycled metabolite related limit could be relevant for specific reactions from central**  
225 **metabolism.** Based on flux values that are either experimentally measured or predicted by  
226 flux balance analysis (FBA), many reactions from the central carbon metabolism are shown  
227 to have lower flux than expected from the kinetics of their immediate enzymes (31). In other  
228 words, these reactions carry fluxes below the first limit identified above in Eq. 2. While



229 substrate limitation and thermodynamic effects can partially explain such lower flux in some  
230 cases (31), the presented theory suggests that limitation due to co-substrate turnover could  
231 also be a contributing factor.

232 To explore this possibility, we re-analysed the flux values compiled previously (31,  
233 32) and focussed solely on reactions that are linked to ATP, NADH, or NADPH pools (see  
234 *Methods* and *Supplementary File 1*). The resulting dataset contained fluxes, substrate  
235 concentrations, and enzyme levels for 45 different reactions determined under 7 different  
236 conditions along with turnover numbers and kinetic constants of the corresponding enzymes.  
237 In total, we gathered 49 combinations of enzyme-flux- $k_{cat}$  values with full experimental data  
238 and 259 combinations with only FBA-predicted flux values. We compared the flux values  
239 that would be expected from the primary enzyme limit identified above, under all conditions  
240 analysed (Fig. 3A), and in addition checked whether the saturation effect of the primary  
241 substrate could explain the difference (Fig. 3B). We found that in both cases, about 80% of  
242 these reactions carry flux lower than what is expected from enzyme kinetics (Fig. S6),  
243 suggesting that the limits imposed by co-factor dynamics might be constraining the flux  
244 further. The low number of the cases where the flux exceeds the limit might be due to  
245 uncertainties in measurement of flux, enzyme or substrate level.

246 To further support the hypothesis that co-substrate turnover dynamics contribute to  
247 the flux limitation, we checked the relation between fluxes and co-substrate pool sizes, which  
248 change among different conditions. For both measured and FBA-predicted fluxes, we find  
249 that several reactions show significant correlation between flux and co-substrate pool size  
250 (see Table S1, *SI* section 8). In the case of FBA-predicted fluxes, however, we note that these  
251 results can be confounded due to additional, flux-to-flux correlations and correlations  
252 between pool sizes and growth rate. Among reactions with measured fluxes, the two reactions  
253 with high correlation to pool size are those mediated by malate dehydrogenase (*mdh*), linked  
254 with NADH pool, and phosphoglycerate kinase (*pgk*), linked with the ATP pool.

255  
256 **Co-substrate cycling allows regulation of branch point fluxes.** In addition to its possible  
257 constraining effects on fluxes, we wondered if co-substrate dynamics can offer a regulatory  
258 element in cellular metabolism. In particular, co-substrate cycling can commonly  
259 interconnect two independent pathways, or pathways branching from the same upstream  
260 metabolite, where it could influence flux distributions among those pathways. To explore this  
261 idea, we considered a model of a branching pathway, with each branch involving a different  
262 co-substrate, *A* and *B* (Fig. 4A and *SI* section 8). This scenario is seen in synthesis of certain  
263 amino acids that start from a common precursor but utilise NADH or NADPH, for example  
264 Serine and Threonine.

265 We hypothesised that regulating the two co-substrate pool sizes,  $A_{tot}$  and  $B_{tot}$ , could  
266 allow regulation of the fluxes on the two branches. To test this hypothesis, we run numerical  
267 simulations with different co-substrate pool sizes and influx rates into the branch point. We  
268 found that the ratio of fluxes across the two branches can be regulated by changing the ratio  
269 of  $A_{tot}$  to  $B_{tot}$  (Fig. 4B). The regulation effect is seen with a large range of  $k_{in}$  values, but the  
270 threshold effect is still present with high enough  $k_{in}$  values leading to loss of steady state and  
271 metabolite build up. In that case, the resulting metabolite build-up can affect either branch  
272 depending on which co-substrate has the lower pool size (see upper corner regions on Fig.  
273 4B). There is also a regime of only the upstream, branch point metabolite building-up, but

274 this happens only when all reactions are considered as reversible and the extent of it depends  
275 on turnover rates of the two co-substrates (Fig S7 and *SI* section 8).

276 In the no-build-up, steady state regime, changing the pool size ratio of the two co-  
277 substrates causes a change in fluxes and metabolite levels, The change in flux ratio is of the  
278 same order as the change in pool size ratio (Fig. 4C & D), while the change in the ratio of  
279 metabolite levels is in general less. This relation between pool size ratio and flux ratio on  
280 each branch is unaffected by the value of  $k_{in}$ . We also evaluated the level of regulation that  
281 can be achieved by varying the turnover rates of *A* and *B*. The flux regulation effect in this  
282 case is weaker, unless the difference in the turnover rates is large and the influx rate is close  
283 to the threshold (Fig. S8).

284

285 **Inter-pathway co-substrate cycling limits maximum influx difference and allows for**  
286 **correlating pathway outfluxes despite influx noise.** We next considered a simplified model  
287 of two independent pathways interconnected by a single co-substrate pool (Fig. 5A and *SI*  
288 section 9). This model can represent several different processes in metabolism, for example  
289 the coupling between the TCA cycle and the respiratory electron transfer chain, through  
290 NADH generation and consumption respectively, or the coupling between the pentose  
291 phosphate pathway and some amino acid biosynthesis pathways (notably Methionine),  
292 through NADPH generation and consumption respectively (S1F). We hypothesised that such  
293 inter-pathway co-substrate cycling might cause the co-substrate related limit to relate to  
294 difference in pathway influxes, rather than input into one pathway, and also balance the  
295 pathway output fluxes against influx fluctuations.

296 To address the first hypothesis, we used analytical methods and explored the relation  
297 between the systems' ability to reach steady state and system parameters. We found that,  
298 indeed, for this coupled system, the ability to reach steady state depends on the influx  
299 difference between two pathways (Fig. S9). This dependence is given by a composite  
300 function of the total pool size and the kinetic parameters relating to pathway-independent  
301 turn-over of the co-substrate (see *SI*, section 10).

302 To test the second hypothesis about the output balancing, we considered the  
303 correlation of the steady-state outputs of the pathways with random fluctuations in their  
304 influx (Fig. 5B). As the pool size decreases, the system reaches a point where there is a  
305 transition from anti-correlation to high correlation in product output (blue to yellow region in  
306 Fig. 5B). At low pool sizes, pathway outputs are fully correlated despite significant  
307 fluctuation in pathway influx (Fig. 5C, D). Within this correlated regime, we identified two  
308 different sub-regimes. The first is a regime where the metabolite concentrations stay  
309 relatively constant despite the influx noise (Fig 5C). This regime arises because the influx  
310 fluctuations are occurring at a much faster rate than the pathway kinetics and the system is  
311 rather non-responsive to influx noise. In a second regime, the influx noise is at time scales  
312 comparable to pathway kinetics. Here, the metabolite concentrations can readily change with  
313 the influx changes, and the system is 'responsive', yet the output levels are still correlated  
314 (Fig. 5D). This regime is directly a result of co-substrate cycling dynamics. Because the  
315 turnover of co-substrate is essentially coupling the two pathways, their outputs become  
316 directly correlated. This effect does not depend on whether pathway reactions are modelled  
317 as reversible or irreversible, but on the rate of the assumed background, i.e. pathway-  
318 independent turnover of the co-substrate (Fig. S10).

319           These results show that coupling by co-substrate cycling can introduce a limit on  
320 influxes of independent pathways or metabolic processes. Furthermore, such coupling can  
321 allow high correlation in the pathway outputs, despite significant noise in the inputs of those  
322 pathways. These effects are most readily seen where the turnover of the coupling co-substrate  
323 by other processes is low. We note that an example case for such a scenario is the coupling of  
324 respiration and oxidative phosphorylation, where transmembrane proton cycling takes the  
325 role of the cycled co-substrate (33).

326

## 327 **CONCLUSIONS**

328 We presented a mathematical analysis of metabolic systems featuring co-substrate cycling  
329 and showed that such cycling introduces a threshold effect on system dynamics. As the  
330 pathway's influx rate,  $k_{in}$ , approaches a threshold value, the steady state concentrations of  
331 metabolites that are upstream of a reaction linked to co-substrate cycling, increase towards  
332 infinity and the system cannot reach steady state. Specifically, for reactions involving co-  
333 substrates, there are two thresholds on influx rate, one relating to the kinetics of the enzyme  
334 directly mediating that reaction, and another relating to the kinetics of the enzymes mediating  
335 the turnover of the co-substrate and the pool size of that co-substrate.

336           This second, additional constraint arising from co-substrate cycling can be directly  
337 relevant for cell physiology. We particularly note that this threshold can be highly dynamic,  
338 and condition- and cell-dependent. When cells have a permanently or occasionally lowered  
339 total co-substrate pool size (i.e. lower  $A_{tot}$ ), or when they are placed under challenging  
340 conditions (e.g. high carbon- or nitrogen-source concentrations) causing higher  $k_{in}$  values  
341 across various pathways, their metabolic systems can be close to the threshold presented here.  
342 While both  $k_{in}$  and  $A_{tot}$  can be adjusted in the long term, for example by reducing substrate  
343 transporter expression or increasing co-substrate biosynthesis, there can be short term impact  
344 on cells experiencing significant flux limitations and metabolite accumulations.

345

346 These results could contribute to our understanding of two commonly observed metabolic  
347 dynamics that arise under increasing or high substrate concentrations, and that are shown to  
348 cause either 'substrate-induced death' (24) or 'overflow metabolism'. The latter usually  
349 refers to a respiration-to-fermentation switch under respiratory conditions (e.g. the Warburg  
350 and Crabtree effects (3, 4, 12, 34)), but other types of overflow metabolism, involving  
351 excretion of amino acids and vitamins, has also been observed (6, 35). Several arguments  
352 have been put forward to explain these observations, including osmotic effects arising from  
353 high substrate concentrations causing cell death and limitations in respiratory pathways or  
354 cell's protein resources causing a respiration-to-fermentation switch (4, 12, 13).

355           Notwithstanding the possible roles of these processes, the presented theory leads to  
356 the hypothesis that both substrate-induced death and metabolite excretions could relate to  
357 increasing substrate influx rate reaching close to the limits imposed by co-substrate  
358 dynamics. There is experimental support for this hypothesis in the case of both observations.  
359 Substrate-induced death and associated mutant phenotypes are linked to the dynamics  
360 associated with ATP regeneration in glycolysis (22-24). Based on that finding, it has been  
361 argued that cells aim to avoid the threshold dynamics through allosteric regulation of those  
362 steps of the glycolysis that involve ATP consumption (23). In the case of respiration-to-  
363 fermentation switch, it has been shown that the glucose influx threshold, at which  
364 fermentative overflow starts, changes upon introducing additional NADH conversion



365 reactions in both yeast and *E. coli* populations (36, 37). In another supportive case, sulfur-  
366 compound excretions are linked to alterations in NAD(P)H pool through changes in the  
367 amino acid metabolism (38, 39).

368 Dynamical thresholds relating to co-substrate pools would be relevant for all co-  
369 substrates, and not just for ATP or NADH, which have been the focus of most experimental  
370 studies to date. We would expect that altering kinetics of enzymes involved in co-substrate  
371 cycling can have direct impact on cell physiology, and in particular on metabolic excretions.  
372 This prediction can be tested by exploring the effect of mutations on enzymes linked to co-  
373 substrate consumption and production, or by altering co-substrate pool sizes and assessing  
374 effects of such perturbations on the dynamics of metabolic excretions. These tests can be  
375 experimentally implemented by introducing additional enzymes specialising in co-substrate  
376 consumption or production (e.g. ATPases, oxidases, or other) and controlling their  
377 expression. It would also be possible to monitor co-substrate pool sizes in cells in real time  
378 by using fluorescent sensors on key metabolites such as ATP or glutamate, or by measuring  
379 autofluorescence of certain pool metabolites, such as NAD(P)H, under alterations to influx  
380 rate of glucose or ammonium.

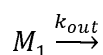
381  
382 Besides acting as a flux constraint, we find that co-substrate pools can also allow for  
383 regulation of pathway fluxes through regulation of pool size or turnover dynamics. We find  
384 that such regulation can take the form of balancing inter-connected pathways, thereby  
385 ensuring correlation between outputs of different metabolic processes, or regulating flux  
386 across branch points. Regulation of fluxes through co-substrate pools can act to adjust  
387 metabolic fluxes at time scales shorter than possible via gene regulation, and possibly at  
388 similar time scales as with allosteric regulation – especially when considering pool size  
389 alterations through exchange among connected pools. Possibility of such a regulatory role has  
390 been indicated experimentally, where total ATP pool size is found to change when yeast cells  
391 are confronted with a sudden increase in glucose influx rate (30). In that study, the change in  
392 the ATP pool is found to link to the purine metabolism pathways, which are linked to several  
393 conserved moieties; GTP, ATP, NAD, NADP, S-adenosylmethionine, and Coenzyme A.  
394 These findings suggest that cells could dynamically alter pool sizes associated with different  
395 parts of metabolism, limiting flux through some pathways, while allowing higher flux in  
396 others, and thereby shifting the metabolites from the latter to the former. This could provide a  
397 dynamic self-regulation and the pool sizes of key co-substrates could be seen as ‘tuning  
398 points’ controlling a more complex metabolic system. We thus propose further experimental  
399 analyses focusing on co-substrate pool sizes and turnover dynamics to understand and  
400 manipulate cell physiology.

401

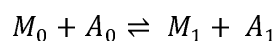
## 402 METHODS

403 **Model of a single reaction with co-substrate cycling.** The metabolic system shown in Fig.  
404 1A, comprises the following biochemical reactions:

405



407



408  
409  
410

$$A_1 \rightleftharpoons A_0 \quad (\text{Eq. 4})$$

411 where metabolites are denoted by  $M_i$  and the different forms of the co-substrate are denoted  
412 by  $A_i$ . We assume additional conversion between  $A_I$  and  $A_0$ , mediated through other  
413 enzymatic reactions. The parameters  $k_{in}$ , and  $k_{out}$  denote the in- and out- flux of  $M_0$  and  $M_I$   
414 respectively, from and to other pathways or across cell boundary. The ordinary differential  
415 equations (ODEs) for the system shown in Eq. 4 (and Fig. 1A), using irreversible Michaelis-  
416 Menten enzyme kinetics would be:

417

$$\frac{dm_0}{dt} = k_{in} - \frac{V_{max} \cdot a_0 \cdot m_0}{K_m + a_0 \cdot m_0}$$

418

419

$$\frac{dm_0}{dt} = \frac{V_{max} \cdot a_0 \cdot m_0}{K_m + a_0 \cdot m_0} - k_{out} \cdot m_1$$

420

421

$$\frac{da_0}{dt} = \frac{V_{max, A_1} \cdot a_1}{K_m, A_1 + a_1} - \frac{V_{max} \cdot a_0 \cdot m_0}{K_m + a_0 \cdot m_0}$$

422

423

$$\frac{da_1}{dt} = \frac{V_{max} \cdot a_0 \cdot m_0}{K_m + a_0 \cdot m_0} - \frac{V_{max, A_1} \cdot a_1}{K_m, A_1 + a_1} \quad (\text{Eq. 5})$$

424

425

426 where  $m_0$  and  $a_0$  denote the concentrations of  $M_0$  and  $A_0$  respectively,  $K_m$  denotes a composite  
427 parameter of the Michaelis-Menten coefficients of the enzyme for its substrates, and  $V_{max}$  is  
428 the total enzyme concentration times its catalytic rate (i.e.  $E = k_{cat} \cdot E_{tot}$ ). We further have the  
429 conservation relation  $a_0 + a_1 = A_{tot}$ , where  $A_{tot}$  is a constant. This assumption would be  
430 justified when influx of any form of the cycled metabolite into the system is independent of  
431 the rest of the metabolic system (see further discussion and analysis in *SI* section 2). The  
432 steady states of Eq. 5 can be found by setting the left side equal to zero and performing  
433 algebraic re-arrangements to isolate each of the variables (see *SI*). The resulting analytical  
434 expressions for steady state metabolite concentration are shown in Eq. 1, and in the *SI* for this  
435 model with reversible enzyme kinetics, as well as for other models.

436

437 **Symbolic and numerical computations.** For all symbolic computations, utilised in finding  
438 steady state solutions and deriving mathematical conditions on rate parameters, we used the  
439 software Maple 2021, as well as theoretical results presented in (40). To run numerical  
440 simulations of select systems, we used Python packages with the standard solver functions.  
441 All numerical simulations were performed in the Python environment. The main model  
442 simulation files relating to Figures 4 and 5 are provided as *Supplementary Files 2* and *3*,  
443 while all remaining simulation and analysis scripts are made available at a dedicated Github  
444 page: <https://github.com/OSS-Lab/CoSubstrateDynamics>.

445

446 **Reaction fluxes and enzyme kinetic parameters.** To support the model findings on co-  
447 substrate pools acting as a possible limitation on reaction fluxes, we analysed measured and  
448 FBA-derived flux data collated previously (31, 32). We focussed our analyses on reactions  
449 involving co-substrates only. We compared measured (or FBA-derived) fluxes to flux  
450 thresholds based on enzyme kinetics (i.e., first condition in Eq. 2). To calculate the latter, we  
451 used data on enzyme kinetics and levels as collated in (31), which is based on the BRENDA

452 database (41) and proteomics-based measurements (42). We note that most available kinetic  
 453 constants for enzymes have been obtained under *in vitro* conditions, which can be very  
 454 different from those of the cytosol (43). When comparing flux levels against co-substrate  
 455 pool sizes, we used the matching, measured pool-sizes from (32). All the data used in this  
 456 analysis is provided in the *Supplementary File 1*, and through a dedicated Github page, which  
 457 contains additional analysis scripts: <https://github.com/OSS-Lab/CoSubstrateDynamics>.

458

## 459 SUPPLEMENTARY FILES

460 **Supplementary Information.** This file contains all of the supplementary figures, the  
 461 mathematical analyses of the reaction systems and corresponding analytical solutions, and the  
 462 descriptions of the simulated models.

463

464 **Supplementary File 1.** Enzyme kinetics, flux, metabolite concentration, and enzyme  
 465 abundance data associated with flux analyses.

466

467 **Supplementary File 2.** Python implementation of branched pathway model, presented in  
 468 Figure 4.

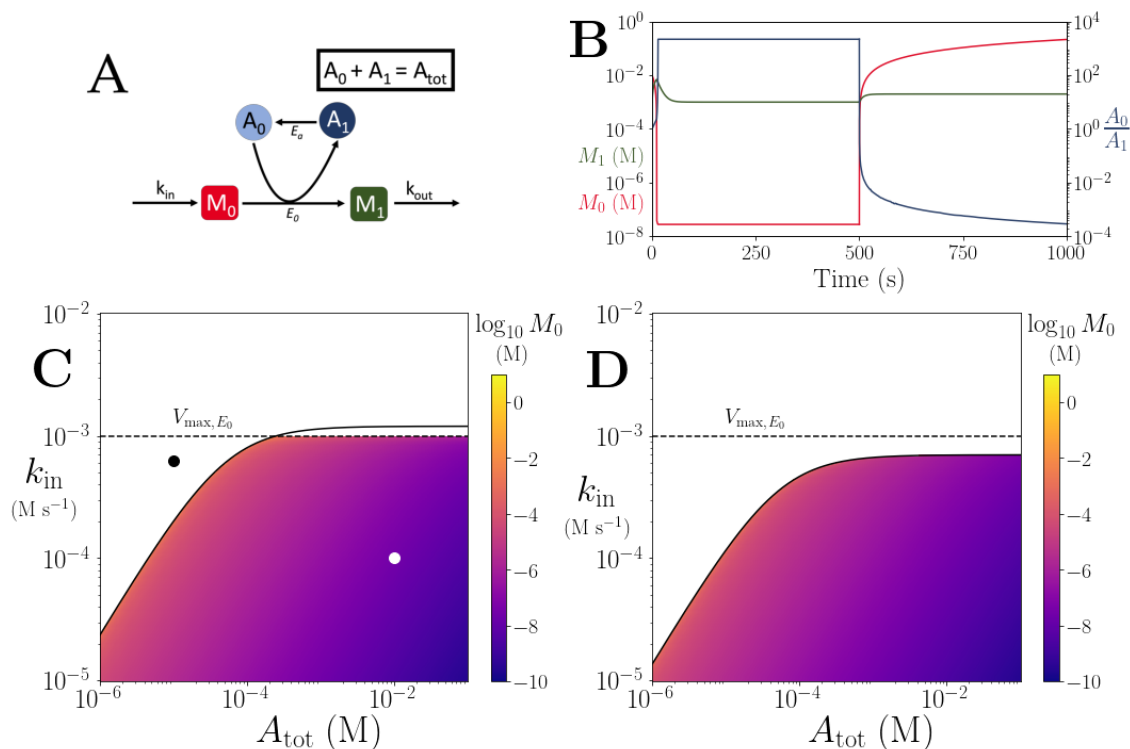
469

470 **Supplementary File 3.** Python implementation of connected pathway model, presented in  
 471 Figure 5.

472

## 473 FIGURES

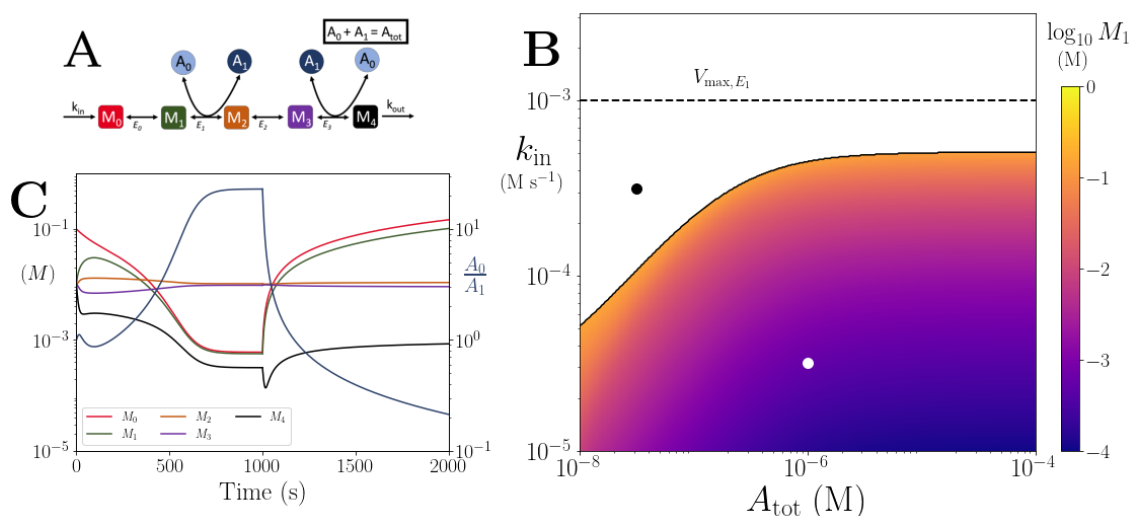
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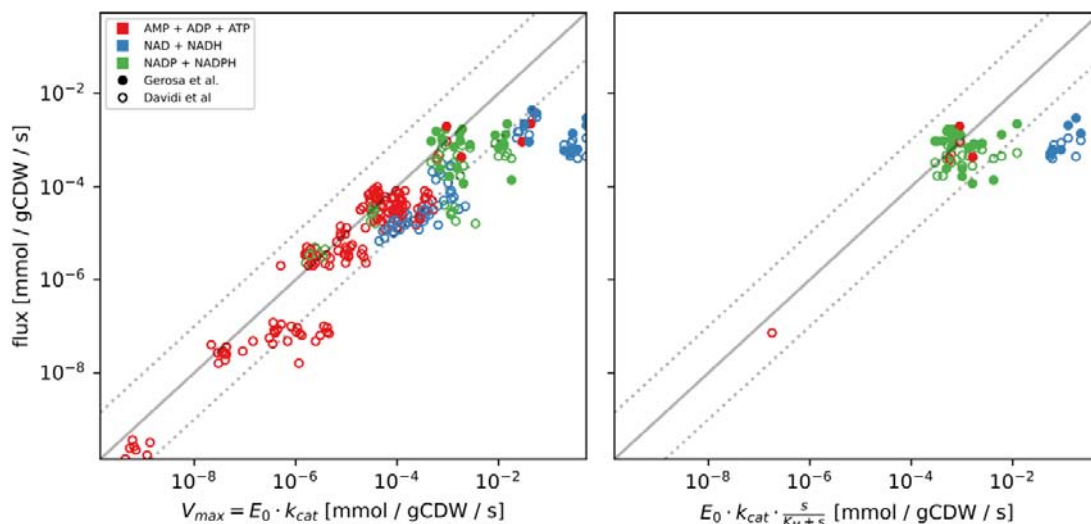
475

476 **Figure 1.** (A) Cartoon representation of a single irreversible reaction with co-substrate  
 477 cycling (see *SI* for other reaction schemes). The co-substrate is considered to have two forms  
 478  $A_0$  and  $A_1$ . (B) Concentrations of  $M_0$  (red) and  $M_1$  (green) and  $A_0/A_1$  ratio (blue) as a function  
 479 of time. At  $t = 500$ , the parameters are switched from the white dot in panel (C) (where a

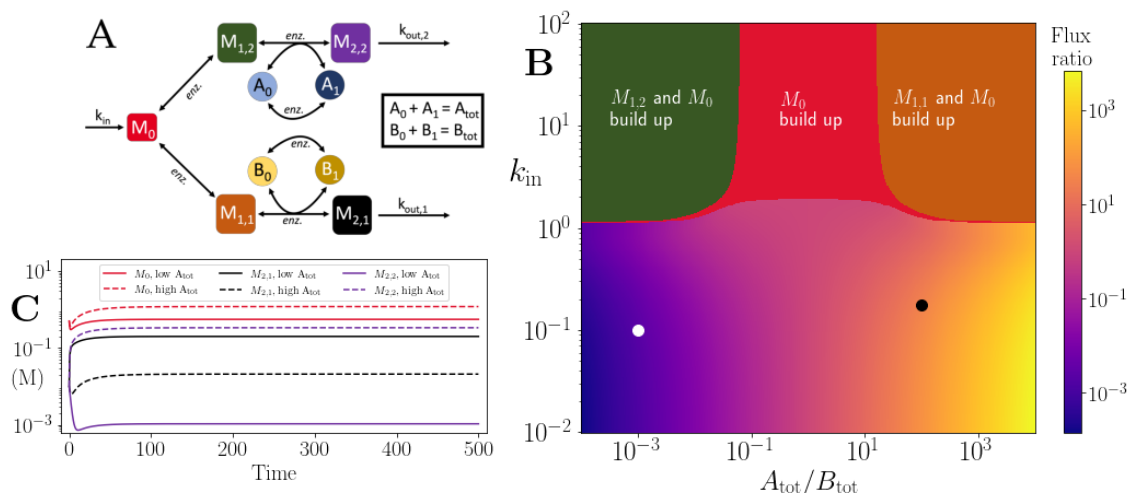
480 steady state exists) to the black dot (where we see continual build-up of  $M_0$  and decline of  $A_0$   
 481 without steady state). **(C & D)** Heatmap of the steady state concentration of  $M_0$  as a function  
 482 of the total co-substrate pool size ( $A_{tot}$ ) and inflow flux ( $k_{in}$ ). White area shows the region  
 483 where there is no steady state. On both panels, the dashed line indicates the limitation from  
 484 the primary enzyme,  $k_{in} < V_{max,E0}$ , and the solid line indicates the limitation from co-substrate  
 485 cycling,  $k_{in} < A_{tot} \cdot V_{max,EA} / (K_{m,EA} + A_{tot})$ . In panel (C), there is a range of  $A_{tot}$  values for which  
 486 the first limitation is more severe than the second. In contrast, in panel (D), the second  
 487 limitation is always more severe than the first. In (B & C) the parameters used for the  
 488 primary enzyme (for the reaction converting  $M_0$  into  $M_1$ ) are picked from within a  
 489 physiological range (see *Supplementary File 1*) and are set to:  $E_{tot} = 0.01\text{mM}$ ,  $k_{cat} = 100 \text{ s}^{-1}$ ,  
 490  $K_{m,E0} = K_{m,EA} = 50\mu\text{M}$ , while  $k_{out}$  is set to  $0.1\text{s}^{-1}$ . The  $E_{tot}$  and  $k_{cat}$  for the co-substrate cycling  
 491 enzyme are 1.2 times those for the primary enzyme. In panel (D) the parameters are the same  
 492 except for and  $E_{tot}$  and  $k_{cat}$  for the co-substrate cycling enzyme, which are set to 0.7 times  
 493 those for the primary enzyme.  
 494



495  
 496 **Figure 2.** **(A)** Cartoon representation of a chain of reversible reactions with co-substrate  
 497 cycling occurring solely inter-pathway. The co-substrate is considered to have two forms  $A_0$   
 498 and  $A_1$ . **(B)** Heatmap of the steady state concentration of  $M_0$  as a function of the total  
 499 metabolite pool size ( $A_{tot}$ ) and inflow rate constant ( $k_{in}$ ). White area shows the region  
 500 where there is no steady state. The dashed and solid lines indicate the limitations arising from  
 501 primary enzyme ( $E_1$  in this case) and co-substrate cycling, respectively, as in Fig. 1. **(C)**  
 502 Concentrations of  $M_{0-4}$ , and  $A_0/A_1$  ratio as a function of time (with colors as indicated in the  
 503 inset). At  $t = 1000$ , the parameters are switched from the white dot in panel (B) (where a  
 504 steady state exists) to the black dot (where we see build-up of all substrates that are produced  
 505 before the first co-substrate cycling reaction, and continued decline of  $A_0$ ). The parameters  
 506 used are picked from within a physiological range (see *Supplementary File 1*) and are set to:  
 507  $E_{tot} = 0.01\text{mM}$ ,  $k_{cat} = 100 \text{ s}^{-1}$ ,  $K_m = 50\mu\text{M}$ , for all reactions, and  $k_{out} = 0.1\text{s}^{-1}$ .  
 508



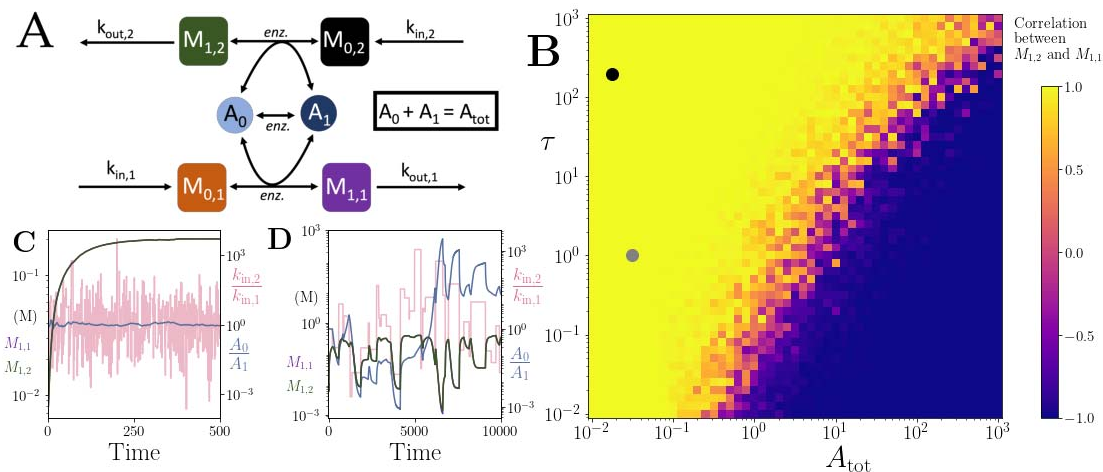
509  
 510 **Figure 3.** (A) Measured and FBA-predicted flux values (from (31, 32)) plotted against the  
 511 calculated primary enzyme kinetic threshold (first part of Eq. 1). Notice that there are 7  
 512 points for each reaction, corresponding to the different experimental conditions under which  
 513 measurements or FBA modelling was done (see *Supplementary File S1* for data, along with  
 514 reaction names and metabolites involved). (B) Measured flux values (from (31, 32)) plotted  
 515 against the calculated primary enzyme kinetic threshold (first part of Eq. 1) adjusted by  
 516 substrate affinity of the enzyme. Note that the flux data shown here is a subset of the flux  
 517 data presented in (A), focusing only on those where the main substrate concentration was  
 518 experimentally measured and the relevant  $K_m$  is known. For both panels, the solid line  
 519 indicates the equivalence of the two values and the dashed lines indicate 10% interval on this,  
 520 as a guide to the eye. Point color indicates the nature of co-substrate involved and fill state  
 521 indicates the data source (as shown on the inset).  
 522



523  
 524 **Figure 4:** (A) Cartoon representation of two branching pathways from the same upstream  
 525 metabolite. The two branches are linked to separate co-substrate pools, A and B. Note that  
 526 pathway independent turnover of the co-substrates is included in the model (see  
 527 *Supplementary File 2*). (B) The pathways' flux ratio (i.e. flux into  $M_{2,2}$  divided by flux into  
 528  $M_{2,1}$ ) shown in colour mapping, against the ratio of co-substrate pool sizes,  $A_{tot}$  and  $B_{tot}$ , and  
 529 the influx rate,  $k_{in}$ , into the upstream metabolite. In the block colour areas, the system has no



530 steady state and the indicated metabolite(s)  $M_0$  and one of the metabolites  $M_{1,2}$  or  $M_{1,1}$   
 531 accumulate towards infinity. (C) Concentrations of upstream and branch-endpoint  
 532 metabolites over time, coloured as shown in the inset of the panel. The solid lines show  
 533 results using parameters indicated by the white dot in panel (B), where  $B_{tot} > A_{tot}$ , while the  
 534 dashed lines show results using parameters indicated by the black dot in panel (B), where  $A_{tot}$   
 535  $> B_{tot}$ . For both simulations, all kinetic parameters are arbitrarily set to 1, apart from the  
 536 pathway-independent co-substrate recycling ( $V_{max,Ea}$ ) that is set to 10 (see *Supplementary File*  
 537 2).  
 538



539  
 540 **Figure 5:** (A) Cartoon representation of two pathways coupled via the same co-substrate  
 541 cycling. The two forms of the co-substrate are indicated as  $A_0$  and  $A_1$ . It is converted from  $A_0$   
 542 to  $A_1$  on the lower pathway, and from  $A_1$  to  $A_0$  in the upper pathway. The presented results are  
 543 for a model with reversible enzyme kinetics, while the results from a model with irreversible  
 544 enzyme kinetics are shown in Fig. S9. (B) Correlation coefficient of the two pathway product  
 545 metabolites,  $M_{1,2}$  and  $M_{1,1}$ , as a function of the total amount of co-substrate ( $A_{tot}$ ) and the  
 546 extent of fluctuations in the two pathway influxes,  $k_{in,1}$  and  $k_{in,2}$ . The influx fluctuation is  
 547 characterised by a waiting time that is exponentially distributed with mean  $\tau$ , after which the  
 548 log ratio of the  $k_{in}$  values is drawn from a standard normal distribution. The mean of the  $k_{in}$   
 549 values is set to be 0.1 and the pathway-independent cycling occurs at a much lower rate  
 550 compared to the other reactions (see *Supplementary File 3*). (C) Concentrations of  
 551 metabolites  $M_{1,2}$  (green) and  $M_{1,1}$  (magenta), pathway influx ratio (pink), and  $A_0/A_1$  ratio  
 552 (blue) as a function of time. The simulation is run with parameters corresponding to the grey  
 553 dot in (B) where the products are correlated, and the rate of  $k_{in}$  fluctuations is on a similar  
 554 timescale to the other reactions. The system is largely unresponsive to the noise. (D)  
 555 Concentrations of metabolites  $M_{1,2}$  (green) and  $M_{1,1}$  (magenta), pathway influx ratio (pink),  
 556 and  $A_0/A_1$  ratio (blue) as a function of time. The simulation is run with parameters  
 557 corresponding to the black dot in (B) where the products are correlated, but the fluctuations  
 558 in  $k_{in}$  values occur at a much lower rate than the other reactions. For both simulations, all  
 559 kinetic parameters are arbitrarily set to 1, apart from the pathway-independent co-substrate  
 560 recycling ( $V_{max,Ea}$ ) that is set to 0.01 (see *Supplementary File 3*).  
 561

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