**SUPPLEMENTARY MATERIALS**

**Mathematical Analysis of Robustness of Oscillations in Models**

**of the Mammalian Circadian Clock**

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# Table S1. Definitions of the dynamical variables in the models.

|  |  |
| --- | --- |
| **Variable** | **Physical Meaning** |
| *M* | *PER* mRNA |
| *Pi* | Cytoplasmic PER species |
| *P* | Nuclear PER |
| *A* | Nuclear BMAL |
| *R* | Nuclear ROR |
| *V* | Nuclear REV-ERB |

# Table S2. Definitions of the dimensionless parameters in the models.

|  |  |
| --- | --- |
| **Parameter** | **Definition** |
| *K*d | Dissociation constant of the PER:BMAL complex |
| *K*A | Dissociation constant of the BMAL:Ebox complex |
| *K*m | Michaelis constant for the degradation of nuclear PER |
| *β*max | Maximum rate of degradation of nuclear PER |
| *α* | Maximum rate of transcription of *Per* gene |
| *A*T | Total BMAL concentration (bound + unbound) |
| *A*MAX | Maximum concentration of BMAL |
| *V*MAX | Maximum concentration of REV-ERB |
| *R*MAX | Maximum concentration of ROR |
| *δ* | Rate constant for turnover of BMAL, REV-ERB and ROR |

# Table S3. Parameter values used in the model simulations in Figure 3.

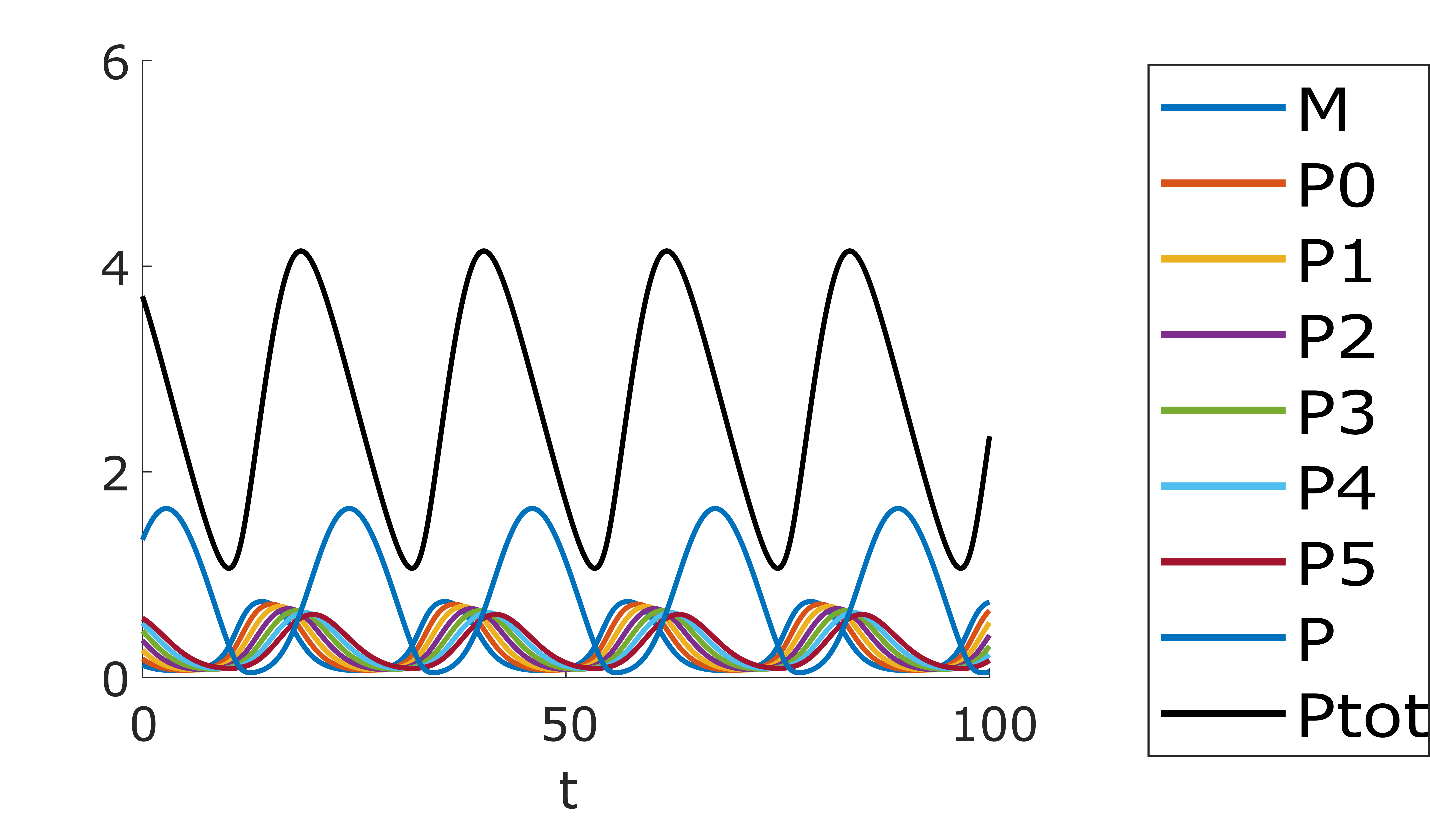
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Param** | **SNF**  **(0LN)** | **SNF**  **(0MN)** | **SNF**  **(1LN)** | **SNF**  **(1MN)** | **SNF**  **(2LN)** | **SNF**  **(2MN)** |
| *K*d | variable | | | | | |
| *K*A |  |  | 0.1 | 0.1 | 0.1 | 0.1 |
| *K*m |  | 0.1 |  | 0.1 |  | 0.1 |
| *A*T | variable | | | | | |
| *β* |  | 4 |  | 4 |  | 4 |
| *φ* | 1 | 1 | 1 | 1 | 1 | 1 |

# Table S4. Parameter values used for the model simulations in Figures 4 and 5.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Panel: | 4a | | | 4b | | |
| **Param** | **SNF**  **(0L8)** | **NNF**  **(0L8)** | **PNF**  **(0L8)** | **SNF**  **(0M8)** | **NNF**  **(0M8)** | **PNF**  **(0M8)** |
| *K*d | 0.001 | 0.001 | 0.001 | 0.1 | 0.1 | 0.1 |
| *K*A |  |  |  |  |  |  |
| *K*m |  |  |  | 0.1 | 0.1 | 0.1 |
| *β* |  |  |  | 4 | 4 | 4 |
| *A*T | 0.3 |  |  | 0.3 |  |  |
| *A*MAX |  | 0.65 | 0.55 |  | 0.70 | 0.54 |
| *δ* |  | 0.2 | 0.2 |  | 0.2 | 0.2 |
| *V*MAX |  | 5 |  |  | 5 |  |
| *R*MAX |  |  | 5 |  |  | 5 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Panel: | 4c, 5(a,b,c) | | | 4d | | |
| **Param** | **SNF**  **(1M8)** | **NNF**  **(1M8)** | **PNF**  **(1M8)** | **SNF**  **(2M8)** | **NNF**  **(2M8)** | **PNF**  **(2M8)** |
| *K*d | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *K*A | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *K*m | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| *β* | 4 | 4 | 4 | 4 | 4 | 4 |
| *A*T | 0.3 |  |  | 0.3 |  |  |
| *A*MAX |  | 0.70 | 0.52 |  | 0.82 | 0.48 |
| *δ* |  | 0.2 | 0.2 |  | 0.2 | 0.2 |
| *V*MAX |  | 5 |  |  | 5 |  |
| *R*MAX |  |  | 5 |  |  | 5 |

# Figure S1. Simulation results of SNF(1M8) model.

****

*P*tot = *P*0+*P*1+*P*2+*P*3+*P*4+*P*5+*P*. All variables, including time *t*, are dimensionless. *K*d = 0.1, *A*T = 0.5, *φ* = 1, and the remaining parameters follow the case SNF(1M8) in Table S4.

May need to replace this figure with the case AT=2 to get a better fit to measured # BMAL molecules in cell.

# Goodwin’s model

To account for observations of periodic enzyme synthesis in bacteria [1], Brian Goodwin [2, 3] presented the following model for the periodic synthesis of an enzyme Y from its mRNA X, where mRNA synthesis is inhibited by a repressor Z that is the product of the catalytic action of Y:

|  |  |  |  |
| --- | --- | --- | --- |
|  |  |  | (1) |
|  |  |  |  |
|  |  |  | (2) |
|  |  |  |  |
|  |  |  | (3) |

In Eq. (1), the factor is the probability that the promoter region of the gene encoding X is not bound to Z, its repressor, and *α*1 is the maximum rate of synthesis of X by the gene. The other terms in these equations correspond to first-order rate laws for production and removal of X, Y and Z. In this tableau, Goodwin’s equations are written in two equivalent forms. On the left, the equations are written in terms of the original dimensional variables: concentrations *X*, *Y* and *Z* (nM) and time *T* (h); on the right, in terms of the ‘dimensionless’ variables , , , dimensionless time , and a dimensionless parameter . In deriving these dimensionless equations, we have assumed (as have all authors in the past) that , which serves to maximize the oscillatory potential of the model [4, 5]. In Goodwin’s version of a three-component negative-feedback loop, the repression of gene transcription by Z is modeled by a Hill function with exponent *p*. Underlying this function is the supposition that the gene encoding X is turned off when *p* molecules of Z bind cooperatively to its promoter region (or, equivalently, when *p* molecules of Z bind cooperatively to an activator of gene transcription and shut it off).

A problem with Goodwin’s model. J.S. Griffith [6] was first to point out that Goodwin’s equations (1)-(3) admit oscillatory solutions only if , a very restrictive condition, because in experimental studies it is rare that more than 3 or 4 protein molecules bind cooperatively to DNA regulatory sequences [7]. This condition becomes even more restrictive if [4].

One solution: a longer feedback loop. The restriction *p* > 8 can be ameliorated by lengthening the feedback loop: if *n* = number of variables in the feedback loop, then the necessary condition for oscillations becomes . For example, for *n* = 8, the condition is *p* > 1.88. Longer loops (*n* > 3) correspond to inserting more than one intermediate (say, Y0, Y1, …, Y*n*−3) between X (mRNA) and Z (feedback component). This is quite reasonable, considering that PER protein has multiple phosphorylation sites [8]. Each intermediate, Y*j*, then denotes cytoplasmic PER phosphorylated on *j* sites, *j* = 0, 1, …, *J*. Eventually, the fully phosphorylated form, Y*J*, is transported into the nucleus and becomes Z. In this case, Goodwin’s dimensionless differential equations become Eqs. (4)-(7).

|  |  |  |
| --- | --- | --- |
|  |  | (4) |
|  |  |  |
|  |  | (5) |
|  |  |  |
|  |  | (6) |
|  |  |  |
|  |  | (7) |

Exactly the same equations can be derived by assuming a distributed time lag between *x* and *z* [9]

|  |  |  |
| --- | --- | --- |
|  |  | (8) |

Introduce the new variables

|  |  |  |
| --- | --- | --- |
|  |  | (9) |

For *j* = 0, we have Eq. (5) for *dy*0/*dt*. For *j* ≥ 1, *Gj*(0) = 0 and d*Gj*(*u*)/d*u* = *Gj*−1(*u*)− *Gj*(*u*); so we have Eq. (6) for *dy*j/*dt* for and Eq. (7) for *dz*/*dt*.

A second solution: Michaelis-Menten degradation of Z. In 1982 Bliss, Painter and Marr [10] proposed to replace the first-order degradation of Z, by a Michaelis-Menten rate law, where is the ‘Michaelis’ constant of the enzyme-catalyzed reaction and is the ‘*V*max’ of the reaction. With this change, the Goodwin model can exhibit limit cycle oscillations even for *p* = 1 [10]. The substitution of Michaelis-Menten rate laws for the first-order kinetic terms in Eqs. (1)-(3) has been exploited by many authors [11-13] to increase the robustness of their models of circadian rhythms.

# Kim & Forger’s extended models

In addition to the SNF model, Kim & Forger proposed two extended models, in which the core negative feedback loop involving PER and BMAL1 is supplemented with (either) an additional negative feedback from REV-ERB on transcription of the *Bmal1* gene (Figure 2b) (or) an additional positive feedback from ROR on transcription of the *Bmal1* gene (Figure 2c) [14]. Both extended models include the ODEs of the core SNF model.

Kim-Forger NNF Model. Equations (1)-(4) of the main text, plus

|  |  |  |
| --- | --- | --- |
|  |  | (10) |
|  |  | (11) |

where *V* is the (scaled) concentration of REV-ERB, *V*maxis the maximum achievable concentration of REV-ERB, *V*o is the REV-ERB concentration that would result in *A*T = 1 at steady state, and *δ* is a rate constant that sets the time scale for the feedback loop.

Kim-Forger PNF Model. Equations (1)-(4) of the main text, plus

|  |  |  |
| --- | --- | --- |
|  |  | (12) |
|  |  | (13) |

where *R* is the (scaled) concentration of ROR, and *R*max, *R*o and *δ* are defined similarly as in the NNF equations. For simulations of the NNF and PNF models, Kim & Forger chose *δ* = 0.2, *V*max = *R*max = 5, and they adjusted *V*o and *R*o to make the NNF and PNF models have the same average activator concentration, <*A*T>, as the SNF model.

# Non-dimensionalization of the modified Kim-Forger equations

The models presented in this paper, as well as Kim and Forger’s original models, were cast in non-dimensional form before simulation and analysis. For example, we show how to non-dimensionalize the SNF(1M8) model:

|  |  |
| --- | --- |
| **Dimensional Equations** | **Non-dimensional Equations** |
|  |  |
|  |  |
|  |  |
|  |  |
|  |  |
|  |  |
|  |  |
|  |  |
|  |  |

where we have already set , because this constraint makes oscillations most likely. The purpose of non-dimensionalization is to ‘scale away’ as many of the kinetic parameters as possible, to reduce the dimensionality of the space of independent parameters. To this end, we make the following change of variables from ‘hat-wearing’ variables (carrying physical units) to their respective dimensionless versions:

, , , …, , ,

The dimensionless ODEs (above right) are governed by five dimensionless parameters:

, , , , and .

# ~~Estimating the scaling factor~~ *~~P~~*~~\* for the SNF(1M8) model~~

~~As in the main text, we estimate the scaling factor~~ *~~P~~*~~\* from the fact that there are a maximum of ~30,000 molecules of PER in a mammalian cell [15]. For the SNF(1M8) model, the total number of PER protein in the cell is~~

|  |  |  |
| --- | --- | --- |
|  |  | ~~(14)~~ |

~~where~~ *~~V~~*~~N~~ ~~and~~ *~~V~~*~~C~~ ~~are the volumes of the nucleus and cytoplasm. Plugging into Eq. (14) the non-dimensionalization factors, we find that~~

|  |  |  |
| --- | --- | --- |
|  |  | ~~(15)~~ |

~~For the case of a simple chain of phosphorylation reactions, we have~~ *~~α~~*~~3~~ ~~=~~ *~~α~~*~~4~~ ~~= … =~~ *~~α~~*~~7~~ ~~=~~ *~~β~~*~~1~~~~. Furthermore, since P~~~~5~~ ~~is transported into the nucleus, . In this case Eq. (15) becomes~~

|  |  |  |
| --- | --- | --- |
|  |  | ~~(16)~~ |

~~From simulations of the model, we find that the sum of nondimensional PER species is ~5 at the peak of its oscillation (Figure S1). Using #PER molecules per cell ≈ 30,000 molecules and nuclear volume = 500 fL, we estimate ≈ 20 nM.~~

~~For the simulations in Figure S1,~~ *~~K~~*~~d~~ ~~= 0.1 and~~ *~~A~~*~~T~~ ~~≈ 2; so 2 nM and 40 nM. The value of corresponds to ~13,000 BMAL molecules in a nucleus of volume 500 fL. The observed number is ~25,000 molecules of BMAL in mammalian cells [15].~~

# ~~Estimation of a physically realistic value for~~

~~To determine a physically realistic value for the dissociation constant, , we must estimate the rate constants for binding and unbinding of the PER:BMAL complex. The upper limit for~~ *~~k~~*~~bind~~ ~~is set by the Smoluchowski rate constant [16] for the diffusion-limited collision of two spherical molecules with diffusion coefficients~~ *~~D~~*~~1~~~~and~~ *~~D~~*~~2~~~~, and radii~~ *~~R~~*~~1~~~~and~~ *~~R~~*~~2~~~~:~~

~~, where (17)~~

~~and~~ *~~N~~*~~A~~ ~~is Avogadro’s number. For reactant molecules with similar sizes and diffusion coefficients, Eq. (17) can be simplified to~~

|  |  |  |
| --- | --- | --- |
|  | ~~;~~ | ~~(18)~~ |

~~hence, the Smoluchowski rate constant for the association of two proteins with radii 2 nm and diffusion constants of 15 μm~~~~2~~~~s~~~~−1~~ ~~would be ~10~~~~9~~ ~~M~~~~−1~~~~s~~~~−1~~~~. Smoluchowski’s equation gives the maximum possible binding rate constant, when every collision between reactants leads to binding. For most molecules, especially proteins, the probability of reaction upon collision is much lower, because the proteins’ binding sites are likely limited to a fraction of the total surface area and successful binding also depends on details of molecular forces at atomic scales. The typical range of measured binding rates between proteins is 0.5‒5×10~~~~6~~ ~~M~~~~−1~~~~s~~~~−1~~ ~~[17].~~

~~The rate constant,~~ *~~k~~*~~unbind~~~~, for the dissociation of PER:BMAL complexes can be estimated from the residence time of the complex, which is likely to be on the order of minutes. So, 0.001 s~~~~−1~~ ~~<~~ *~~k~~*~~unbind~~ ~~< 0.01 s~~~~−1~~~~. With the previously derived diffusion-limited binding constant,~~ *~~k~~*~~bind~~ ~~≈ 1 nM~~~~−1~~~~s~~~~−1~~~~, the absolute lower limit on the dissociation constant is is in the range 10~~~~−3~~ ~~to 10~~~~−2~~ ~~nM. However, because~~ *~~k~~*~~bind~~ ~~is typically at least three orders of magnitude lower than the Smoluchowski limit [17], a more realistic range for is 1 to 10 nM. Recent measurements of the binding of PER:CRY to BMAL:CLOCK [18] suggest that is considerably larger than 10 nM, so we propose that   
10 nM < < 100 nM is a reasonable range for the value of this binding constant. Our estimated value 2 nM for the simulations in Figure S1 lies close to this range.~~

# Deriving the rate laws for *PER* transcription

BMAL:CLOCK binds to many E-box sequences throughout the mammalian genome, and PER:CRY binds to both free and E-box-bound BMAL:CLOCK complexes. Let E*i*, *i* = 1, …, Ω, denote all the E-box sequences that bind all four proteins BMAL, CLOCK, PER and CRY, where Ω ≈ 1500 according to ChIP-seq data [19]. The total concentrations of BMAL:CLOCK (A) and PER:CRY (P) dimers are:

|  |  |  |
| --- | --- | --- |
|  |  | (10) |

|  |  |  |
| --- | --- | --- |
|  |  | (11) |

We are assuming that the synthesis and degradation of proteins are much slower reactions than the association and dissociations of proteins in a complex, so the total amounts of A and P in the system can be treated as constants on the time scale of the binding and unbinding reactions.

Because the total number of E-boxes (~1500) is considerably less than the total number of BMAL:CLOCK and PER:CRY complexes (~25,000 and ~30,000, respectively [15]), we can reasonably neglect the E-box-bound forms of the protein complexes, and assume that

|  |  |  |
| --- | --- | --- |
|  |  | (12) |

|  |  |  |
| --- | --- | --- |
|  |  | (13) |

To derive an expression for the rate of *PER* transcription, we need to estimate the fraction of E-boxes bound to BMAL:CLOCK but not to PER:CRY, i.e., [A:E*p*]/[E*p*]T, where *p* is the index corresponding to E-boxes driving *PER* gene expression. To this end, we consider the equilibrium binding reactions:

|  |  |  |
| --- | --- | --- |
|  |  | (14) |

|  |  |  |
| --- | --- | --- |
|  |  | (15) |

|  |  |  |
| --- | --- | --- |
|  |  | (16) |

|  |  |  |
| --- | --- | --- |
|  |  | (17) |

The equilibrium dissociation constants of Reactions (14)-(17) are defined by

|  |  |  |
| --- | --- | --- |
|  |  | (18) |

The principle of detailed balance at equilibrium requires that

|  |  |  |
| --- | --- | --- |
|  |  | (19) |

That is, the dissociation constants for Reactions (14)-(17) satisfy

|  |  |  |
| --- | --- | --- |
|  |  | (20) |

Taking Eq. (20) into account, we are left with only three independent chemical equilibrium equations. One of these equations is

|  |  |  |
| --- | --- | --- |
|  |  | (21) |

which can be solved for the unknown concentration of A:P,

|  |  |  |
| --- | --- | --- |
|  |  | (22) |

Meanwhile, the total number of *PER* E-boxes, [E*p*]T = [E*p*] + [A:E*p*] + [P:A:E*p*], can be written as:

|  |  |  |
| --- | --- | --- |
|  |  | (23) |

which can be rearranged to give the probability that a *PER* gene is being transcribed:

|  |  |  |
| --- | --- | --- |
|  |  | (24) |

Plugging the definition of *K*dAP1 (Eq. (18)) into Eq. (24) yields

|  |  |  |
| --- | --- | --- |
|  |  | (25) |

First Case. PER:CRY binds equally strongly to free- and E-box-bound BMAL:CLOCK, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (26) |

In this case, Eq. (25) becomes

|  |  |  |
| --- | --- | --- |
|  |  | (27) |

Plugging Eq. (12) into Eq. (27) yields Rate Law 1:

|  |  |  |
| --- | --- | --- |
|  |  | (28) |

Second Case. PER:CRY binds equally strongly to free- and E-box-bound BMAL:CLOCK, Eq. (26) (First Case above), and at the same time, BMAL:CLOCK saturates the *PER* E-box, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (29) |

In this case, Eq. (28) becomes Rate Law 0 in the original Kim-Forger model:

|  |  |  |
| --- | --- | --- |
|  |  | (30) |

Third Case. BMAL:CLOCK cannot or can hardly bind PER:CRY and E-box simultaneously, i.e.,

|  |  |  |
| --- | --- | --- |
|  |  | (31) |

In this case, Eq. (25) gives rise to Rate Law 2:

|  |  |  |
| --- | --- | --- |
|  |  | (32) |

Electron microscopy studies by Aryal et al. have shown that PER:CRY::BMAL:CLOCK complexes bind to E-boxes [20], indicating that rate laws 0 and 1 are to be preferred to rate law 2.

# Simulation methods

Bifurcation diagrams in Figures 3-5 were produced using XPP-AUTO. To recreate these diagrams, the integration method should be changed to stiff in XPP by executing Numerics > Method > Stiff. The default simulation parameters (Tolerance = 0.001, Minimum step = 1e-12, Maximum step = 1) can be used. To trace a bifurcation curve, a stable steady-state for the parameter of interest should be imported with Sing pts > Go > Import. With the steady-state imported, AUTO is launched with File > Auto. In AUTO, the numerical parameters for the bifurcation calculations must be set in the Numerics menu as such:

Ntst = 30

Nmax = 2000

NPr = 0

Ds = ±0.002 (depending on integration direction)

Dsmin = 0.0001

EPSL = 0.00001

Dsmax = 0.05

Par Min and Par Max should be set according to the axes on the bifurcation diagram. All other numerical parameters can be left at their default values.

The first step is to compute a one-parameter bifurcation diagram. First, create the plotting plane using Axes > hi-lo and then follow the imported steady-state with respect to one parameter by the command Run > Steady state in AUTO. Next, Grab a Hopf bifurcation point and change the axes to a two-parameter view using Axes > Two par. Then trace the two-parameter bifurcation curve using Run > Two Par. Bifurcation points can then be exported from AUTO using File > Write pts. We used MATLAB to plot these curves.

AUTO can also be used to trace fixed-period trajectories, as in Figure 5. To do this, the desired periods should be entered into the Usr period menu in AUTO before computing the one-parameter bifurcation diagram. Then, on the two-parameter bifurcation diagram Grab a marked user period (UZ) point and execute Run > Fixed Period. To export the periods, save all information for each point by executing File > All info.

XPP-AUTO Documentation and Installation: <http://www.math.pitt.edu/~bard/xpp/xpp.html>

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