Perkins et al.:

Chemical Hazard Prediction and Hypothesis Testing Using Quantitative Adverse Outcome Pathways

Supplementary Data

Network model

The model is an extension of the steroidogenic model for fathead minnow (FHM) along HPG axis developed by Shoemaker et al. (2010) with the incorporation of a liver compartment from which VTG is synthesized. Gonadotropin releasing hormone (GnRH) is produced in the hypothalamus and activates the transcription of luteinizing hormone (LH) in pituitary via the gonadotropin-releasing hormone receptor (Villeneuve et al., 2012). This is mathematically described in equation S1:

$$\frac{d[L_m]}{dt} = V_{spLH}\frac{[GnRH]^n}{KcpLH + [GnRH]^n + {[T_{ex}]^2}/{K_{inLH}} + {[E2_{ex}]^2}/{K_{inLH}}} - V_{mpLH}\frac{[L_m]}{K_{mpLH} + [L_m]} - K_{dmpLH}[L_m]$$

Where $\frac{d[L_m]}{dt}$ denotes the synthesis rate of LH mRNA pituitary and it is negatively controlled by plasma extradiol ($E2_{ex}$) and testosterone (T_{ex}) (for details see appendix). LH protein is synthesized in pituitary and secreted into blood which carries it to the ovary. Binding to the luteinizing hormone receptor (LHR) initiates a complex signaling cascade in the ovary (involving LH receptor recycling, adenylate cyclase activation, protein kinase A activation, protein kinase A inhibition, and phosphodiesterase activation, as described in detail in Bhalla (2002), Hao et al. (2006), and Shoemaker et al. (2010)). Ultimately, steroidogenic factor 1 (SF1) is activated by the complex signaling cascade and thereby simulating transcription of Steroidogenic Acute Regulatory (StAR) protein.

The StAR protein is responsible for transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Cholesterol is transformed through a series of enzyme-catalyzed reactions leading to the production of all sex steroids considered. Once cholesterol has been processed E2, T, androstenedione (AD), estrone (E1) are secreted into the blood. These hormones are carried by blood to the other organs of the body where they regulate biochemical processes. VTG is a lipoprotein produced in the liver in response to E2 whose production is required for proper oocyte development. The process for transcription of VTG was represented as equation S2:

$$\frac{d[Vtg_m]}{dt} = \frac{1}{\tau_{dm}}\left\{\frac{VspVtg * [E2_{ex}]^2}{KcpVtg^2 + [E2_{ex}]^2} - \frac{VmpVtg * [Vtg_m]}{KmpVtg + [Vtg_m]} - KdmpVtg * [Vtg_m]\right\}$$

Where $\frac{d[Vtg_m]}{dt}$ is synthesis rate of VTG mRNA and controlled by plasma estradiol ($E2_{ex}$), τ_{dm} denotes the time delay response of the transcription process. The process of translation of VTGprotein ($\frac{d[Vtg_p]}{dt}$) was described with a time delay function (τ_{dp}) as follows equation S3:

$$\frac{d[Vtg_p]}{dt} = \frac{1}{\tau_{dp}}\left\{\frac{VprVtg * [Vtg_m]}{KprVtg + [Vtg_m]} - KdprVtg * [Vtg_p]\right\}$$

Recently, Miller et al. (2006) developed a FHM population forecast model in an effort to extrapolate from levels of VTG as a biomarker for fecundity (specifically, the egg production rate) to potential impacts that endocrine disrupting chemicals might have on fish populations. The population model was put to use by integrating the Leslie projection matrix (Leslie, 1945) and the logistic equation S4 (Miller et al., 2007, 2004; Ankley et al., 2008).

$$n_{t+1} = exp\left(\frac{-rP_t}{K}\right)M^* n_t$$

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Here n_{t+1} is the age structure vector population at time $t+1$, n_t is the age structure vector population at time t, r is the intrinsic rate of increase, P_t is the population size at time t, M^* is the Leslie matrix containing fecundity and fertility. Coefficients of fecundity in the Leslie Matrix are adjusted as relative fecundity observed during FAD exposure.

We defined a dynamic steroidogenesis model with a set of ordinary differential equations (ODEs). Dynamics of metabolite events are much slower that transcription, translational and signaling events. Therefore, this is a computationally stiff equation set and the *ode15s*MATLAB solver (Mathworks, Natick, MA, USA) was applied to simulate this network.

Parametric sensitivity analysis

The dynamic steroidogenesis process along PLG axis is represented as ordinary differential equations (ODE) containing 59 states and 122 parameters. The importance of these parameters in determining system behavior was evaluated to identify which mechanisms within the steroidogenesis network model is primary regulators steroid production. Sensitivity analysis is a classic technique to investigate process behavior resulting from parametric perturbations and used to design informative experiments (Yue et al., 2008; Zak et al., 2005) or to study robustness properties of biological systems (Bagheri et al., 2007; Deeley et al., 1975; Stelling, 2004; Taylor et al., 2009). Relative sensitivity is defined as

$$\hat{S} = \frac{dx}{dp} \times \frac{p}{x} = \frac{d\ln(x)}{d\ln(p)}$$

where x is the state vector and p is the parametric vector.

Results

VTG is the precursor of the egg yolk phosphor-protein and is synthesized in liver (Deeley et al., 1975). Two states, transcription and translation of VTG, were accounted for in the liver compartment where E2 is considered a precursor in VTG synthesis (Fig. S1). Experimental observations suggest that the dynamics of VTG synthesis is considerably slower than E2 and T (Villeneuve et al., 2009). Therefore, a time delay was included as vital parameter to account for the time lag between estradiol synthesis and the physiological responses of translation and transcription of VTG.

The GnRH pulse rate and amplitude controlling the response of VTG and other sex hormones was a concern. Inadequate data was available to determine proper values; therefore, we simulated the network to explore the behavior of VTG at different combinations of amplitude (0.0 to 100 nM) and pulse rate (0-120 min) (Fig. S2). It was observed that the VTG response was sensitive towards GnRH amplitude and relatively independent of the pulse rate. We used typical biological GnRH parameters as a forcing function with 10 nM amplitude and a 15 minute pulse rate.

We explored the responses of E2, T, and VTG in the HPG model during an 8 day (198 h) exposure to FAD (3 µg/l and 30 µg/l) followed by removal of FAD from the water medium for another 8 days (Fig. S3; Villeneuve et al., 2009). The results for E2 and T are derived from the study done by Shoemaker et al. (2010). The results showed that the relative levels of E2 and VTG are lower in the presence of FAD with respect to nominal levels. However, the response time of VTG to presence of FAD was slower relative to E2. Interestingly, the relative response of T was higher than nominal levels. The model predictions were found to be consistent with the experimental observations for plasma levels of T and VTG during the 8 day exposure (Villeneuve et al., 2009). The model failed to predict compensatory mechanisms that resulted in E2 predictions that were offset from experimental values at 4 and 8 days. Model predictions were inconsistent with experimental findings of an overshoot response during the early recovery phase. While the model results for relative VTG response were consistent with experimental observations the during FAD exposure phase, experimental VTG levels required substantially more time to achieve control levels than in the model simulation.

Our network model contains 59 states and 122 parameters. Therefore, we used dynamic parametric sensitivity analysis to examine the model for sensitive parameters by varying parametric perturbations over the nominal values (Fig. S4). We found that the most sensitive parameters were 1, 2, 4, 17-20, 43-45, and 51-75. Parameters 1, 2, 4 were related to GnRH, ATP, and total StAR protein concentration, respectively. Other sensitive parameters were related to adenylate cyclase activation and protein kinase-A activation followed by StAR activation processes. Parameters related to the steroid production, VTG production in liver and LH synthesis in pituitary (parameter 94-122) was comparatively less sensitive. However, LH receptor complex (LR, state 2), LH receptor G protein complex (LGGDPGasbg, state 5) were sensitive to parameters related to adenylate cyclase activation, protein kinase A activation and StAR activation. Cyclic AMP recycling process (state 16-21) were also sensitive to parameters ranging from 51 to 75. Inhibinα (state 40-41), LH synthesis rate (state 42-43) and VTG synthesis rate (state 58-59) were also found to be similarly sensitive. Steroid synthesis (state 44-57) was comparatively less sensitive to parameters of other processes and also less sensitive from its own parameters.

The relative parametric sensitivity for VTG transcription process in time domain under exposure of FAD varied on the first day of transcription processes (Fig. S5). However, sensitivity remained essentially constant at later phases (second day onwards) of transcription processes. Results show positive sensitivity for GnRH and ATP concentrations and negative sensitivity for total StAR protein concentration. This process is regulated by AC activation, PKA activation and StAR activation (parameter 51-75). Also, VTG transcription process was found to be sensitive to its own parameters and insensitive to steroid synthesis process parameters except for the parameters related to the E2 and T synthesis.

We linked this network model with the population model to assess population level effects due to FAD exposure. The relationship between relative fecundity, E2, T, and VTG is reported as a simple linear regression model based on the 21-days reproductive studies with different chemical stressors (Miller et al., 2007; Ankley et al., 2008):

$$fecundity = -0.042 + 0.95Vtg \qquad (r^2 = 0.88)$$

$$fecundity = 0.008 + 0.52E2 \qquad (r^2 = 0.58)$$

$$fecundity = 0.032 + 0.457T \qquad (r^2 = 0.32)$$

In this study, we used the correlation between fecundity and VTG (as this correlation has a relatively high confidence level as compared to others) for extrapolation to population level effects. The Leslie matrix generated an intrinsic rate equal to 0.337 and the fecundity coefficient of the Leslie matrix was adjusted according to the relative fecundity calculated from correlation equation. A carrying capacity of 5665 fish/1000m^3 was used as reported by Neil (1979) while a stable age distribution of 4178 fish in the 1 year age class, 1163 fish in the 2 year age class, and 324 fish in the 3 year age class was used (Miller and Ankley, 2004).

We predicted the population trajectories for various levels of constant FAD exposure (Fig. S6). The depressed VTG level emerging from constant FAD exposure resulted in a predicted population decline. Remarkably, the relative population trajectory stabilizes at low population levels (50% of the population is maintained relative to no FAD exposure) for constant exposures of up to 0.5 µg/l of FAD. Also, population trajectory becomes stable but with a considerably lower relative capacity during a consistent exposure to 1 µg/l of FAD (Fig. S6, line D). Concentrations of FAD above 1µg/l produced catastrophic effects resulting in the total collapse of the population (Fig. S6). The model presented here suggests that the relative population would vanish in 20 years, 12 years, and 3 year when fish are exposed to 2 µg/l, 3 µg/l, and 30 µg/l concentrations of FAD, respectively. It is interesting to note here that the population level is highly sensitive to FAD exposure and the significance of this sensitivity is more towards the higher concentrations of FAD exposure. For example: on increasing the FAD concentration 1.5 times, the time of survival decreases by 8 years (lines E and F). Also, the stabilizing nature of normalized population is a vital observation of the study. It gives us an insight into the relative detriment being done by the corresponding FAD exposure concentration.

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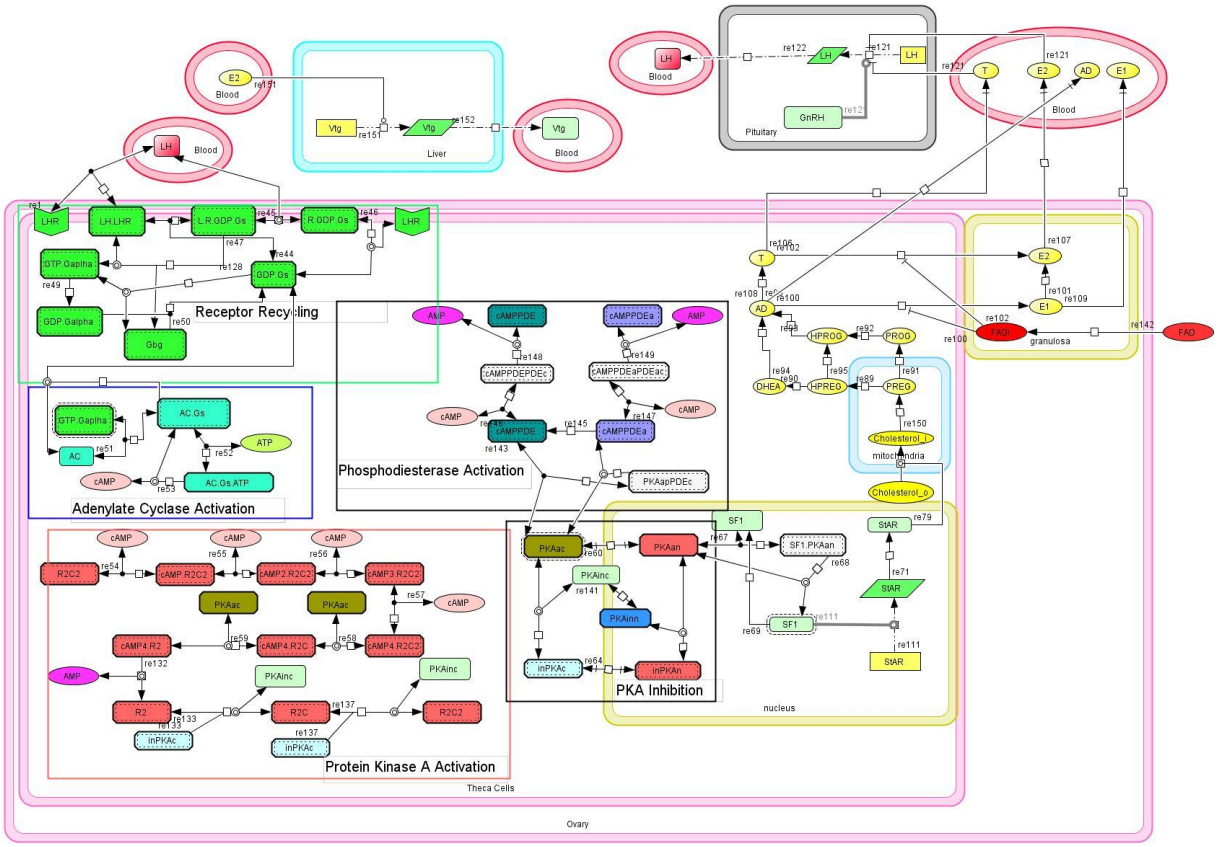

Fig. S1: Schematic of the steroidogenesis pathway along pituitary-liver-gonad (PLG) axis
Lituanizing hormone (LH) synthesized in pituitary which is controled by Gonadotropine releasing hormone (GnRH), Testosterone (T), and estradiol (E2). Vitellogenin (Vtg) is synthesized from liver which is control by E2. Steroidogenesis pathway is activated from LH receptor recycling, adenylate cycle (AC) activation, protein kinase A (PKA) activation, sterogenic factor 1 (SF1) activation, and steroigenic acute regulatory (STAR) gene activation. Cholesterol is the precursor for hormone synthesis which is regulated by STAR protein. Fadrazole (FAD) is a competitive inhibitor which inhibits CYP aromatase.

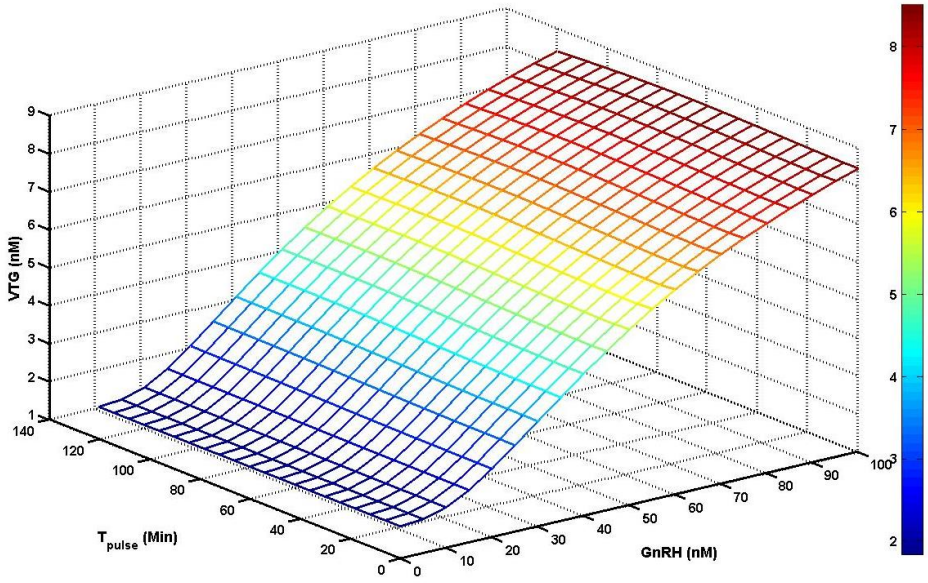

Fig. S2: Solution space for VTG production for different combination of GnRH concentrations and duration of impulse for GnRH
VTG response is robust to the impulse of GnRH, while sensitive the GnRH concentration.

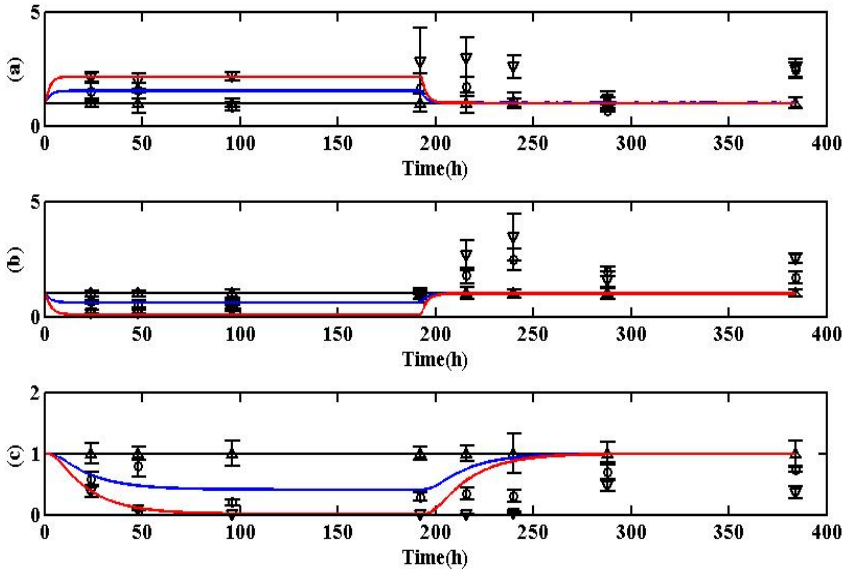

Fig. S3: Responses of the different hormones for 8 days (198 h) exposure of fadrozole (FAD) followed by next 8 days of depuration for *Pimephales promelas*
Lines indicate: black, control; blue, exposure of 3 µg of FAD; red, 30 µg of FAD. Symbols indicate the experimental data points (Reference is to be given). Y axis indicates the normalized value with respect to control. Responses of testosterone (a) and estradiol (b) were faster than vitellogenin (c) under FAD exposure.

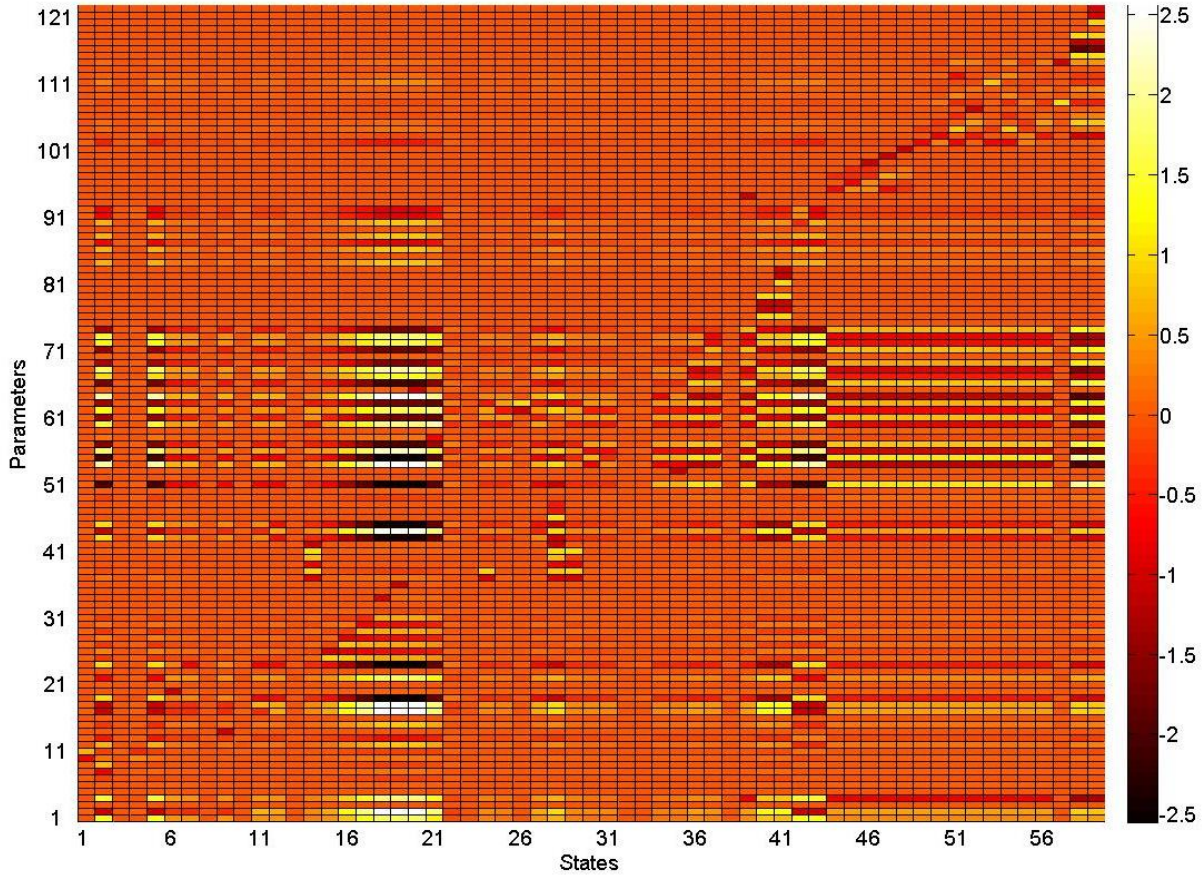

Fig. S4: Relative parametric sensitivity for all states related to steroidogenesis for fathead minnow (*Pimephales promelas*) under exposure of fadrozole

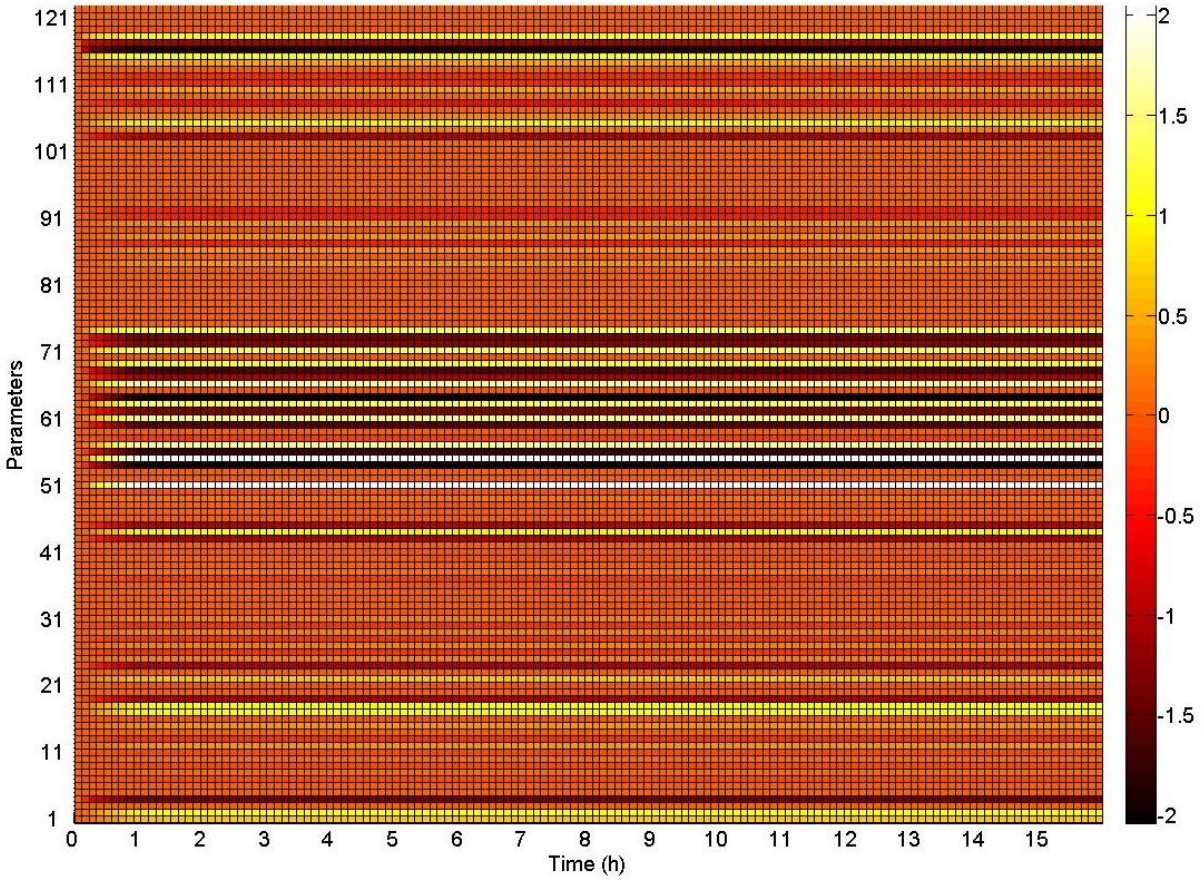

Fig. S5: Relative parametric sensitivity for vitellogenin transcription along exposure time for fathead minnow (*Pimephales promelas*) steroidogenic model under exposure of fadrozole

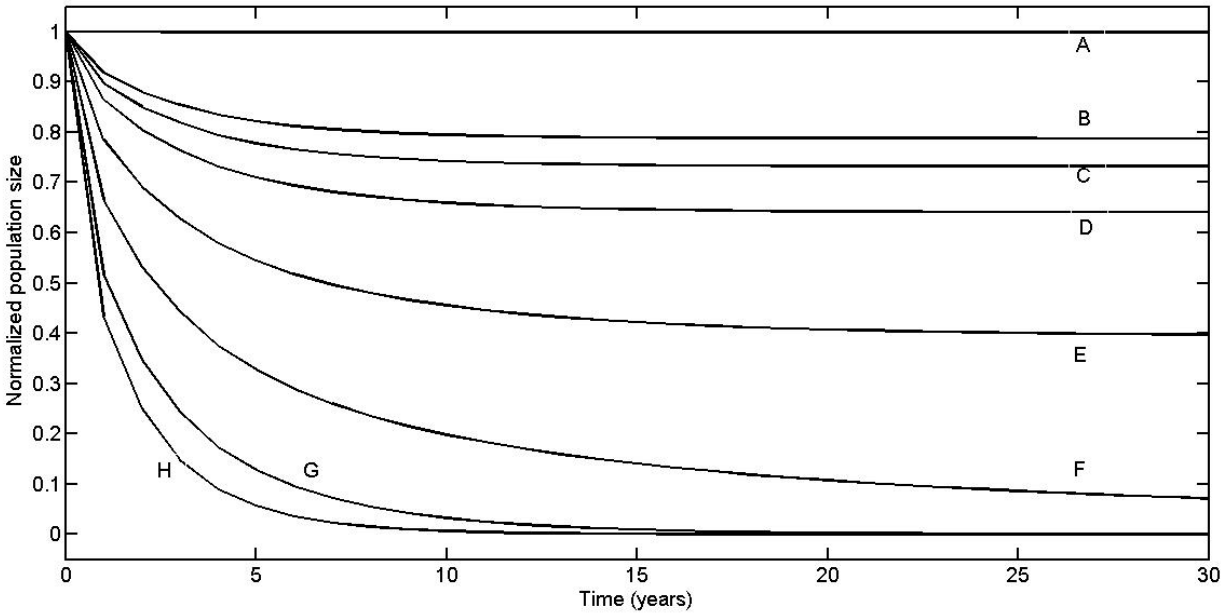

Fig. S6: Relative population trajectory forecasted for fathead minnow (*Pimephales promelas*) under exposure of different levels of fadrazole

(A) No fadrozole, (B) 0.04 µg/l fadrozole, (C) 0.1 µg/l, (D) 0.2µg/l, (E) 0.5 µg/l, (F) 1µg/l, (G) 2 µg/l, (H) 3 µg/l FAD medium. Relative population becomes stable at lower capacity (78%) for fadrozole exposure of 0.04 µg/l while capacity becomes 40% at 0.5 µg/l of fadrozole exposure. Population becomes zero by 15 and 10 years under fadrozole exposure of 2 and 3 µg/l, respectively.