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In silico predicted structural and functional robustness of piscine steroidogenesis

D. Hala*, D.B. Huggett

Institute of Applied Sciences, University of North Texas, Denton, TX 76203, USA

HIGHLIGHTS

- We simulated steroidogenic robustness subject to *in silico* reaction deletions.
- Network was susceptible to deletion of structurally relevant reactions.
- Structurally relevant reactions fitted power law distribution.
- Network robustness was not affected by deletion of high flux reactions.
- High flux reactions were a poor fit for power law distribution.

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ABSTRACT

Assessments of metabolic robustness or susceptibility are inherently dependent on quantitative descriptions of network structure and associated function. In this paper a stoichiometric model of piscine steroidogenesis was constructed and constrained with productions of selected steroid hormones. Structural and flux metrics of this *in silico* model were quantified by calculating extreme pathways and optimal flux distributions (using linear programming). Extreme pathway analysis showed progestin and corticosteroid synthesis reactions to be highly participant in extreme pathways. Furthermore, reaction participation in extreme pathways also fitted a power law distribution (degree exponent $\gamma=2.3$), which suggested that progestin and corticosteroid reactions act as 'hubs' capable of generating other functionally relevant pathways required to maintain steady-state functionality of the network. Analysis of cofactor usage (O_2 and NADPH) showed progestin synthesis reactions to exhibit high robustness, whereas estrogen productions showed highest energetic demands with low associated robustness to maintain such demands. Linear programming calculated optimal flux distributions showed high heterogeneity of flux values with a near-random power law distribution (degree exponent $\gamma \geq 2.7$). Subsequently, network robustness was tested by assessing maintenance of metabolite flux-sum subject to targeted deletions of rank-ordered (low to high metric) extreme pathway participant and optimal flux reactions. Network robustness was susceptible to deletions of extreme pathway participant reactions, whereas minimal impact of high flux reaction deletion was observed. This analysis shows that the steroid network is susceptible to perturbation of structurally relevant (extreme pathway) reactions rather than those carrying high flux.

1. Introduction

The study of metabolic phenotypes is becoming increasingly dependent on thorough quantitative descriptions of network structure and associated function (Kanehisa 2002, 2008; Feist et al., 2009). There is growing interest in identifying underlying 'optimal' behaviors of metabolic systems that may maximize an organism's (or species) fitness and survival success (Ibarra et al., 2002; Smith, 1978).

The structural and functional analyses of metabolic networks are unified by stoichiometric representations of metabolite mass-balancing in interrelated enzyme-catalyzed reactions (Planes and Beasley, 2008; Orman et al., 2011). Represented in this form, various mathematical operations can be used to assess system characteristics. For example, row reduction (using Gaussian elimination) of the stoichiometric matrix to reduced-row-echelon-form enables determination of linearly independent basis vectors that span

* Corresponding author. Tel.: +1 940 206 4973.
E-mail addresses: david.hala@unt.edu (D. Hala), dbhuggett@unt.edu (D.B. Huggett).

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the null space of the matrix (Lay, 1997). The imposition of strict non-negativity constraints on these basis vectors generates a convex polyhedral subspace (or cone) in which vectors generating the edges of the subspace are called extreme pathways (Schilling and Palsson, 1998). These extreme pathways correspond with essential biochemical pathways that balance steady-state system demands of substrate uptake and production. Furthermore, the linear independence of these pathways enables generation of (as non-negative linear combinations) all other functional biochemical pathways comprising the network (Papin et al., 2002, 2004; Price et al., 2002; Wiback and Palsson, 2002; Xi et al., 2009; Llaneras and Pico, 2010).

Additionally, such matrix representations allow the use of linear optimization methods to calculate an optimal solution that maximizes (or minimizes) a system performance measure or objective function (Fell and Small, 1986; Savinell and Palsson, 1992). More specifically, linear programming has been successfully used to predict optimal metabolic flux distributions (i.e., metabolic pathways and associated flux values) required for maximal growth or metabolite productions of various prokaryotic and eukaryotic organisms (Varma and Palsson, 1994; Edwards et al., 2001; Forster et al., 2003; Fong et al., 2005; Yim et al., 2011). The success of this approach relies on the hypothesis that metabolic networks have evolved under selective pressures to produce an optimum metabolic phenotype (Smith, 1978; Nielsen, 2007). In turn, simulation and experimentation (using microbial organisms) show good agreement with this assumption as optimal flux distributions tend to align with maximal biomass (growth) and ATP productions (Edwards et al., 2001; Burgard and Maranas, 2003; Schuetz et al., 2007).

In this paper, we aim to explore the association between network structure and function in reproductive steroidogenesis (or steroid hormone production) of fish. Steroidogenesis comprises the metabolic conversion of cholesterol to various steroid hormones such as: androgens, estrogens, progestins, and corticosteroids (Supplemental 1). These conversions are under the concerted control of various cytochrome P450 and hydroxysteroid dehydrogenase enzymes, and the steroid hormones produced play critical roles in establishing and maintaining normal reproductive function (Matty, 1985; Kime, 1993; Omura and Morohashi, 1995). We pose the following broad question: do structural or flux properties of steroidogenic reactions lend to robustness when subject to perturbation?

In order to address this question, an *in silico* model of steroidogenesis was constructed by stoichiometrically reconstructing the consumption/production of 116 metabolites in 120 steroid enzyme catalyzed reactions. Firstly, this *in silico* model was treated as a homogenous system with experimentally quantified system demands of steroid metabolite uptake and production rates not taken into account. For this system, the total numbers of extreme pathways utilizing each steroidogenic reaction were determined to give a measure of reaction importance as high extreme pathway participation implies functional importance in the network, as their linear independence can help derive all other functioning pathways comprising the network. Subsequently, the model was constrained with experimentally determined production rates (or system demands) of ten steroid hormones using *ex vivo* culture of ovary tissues from sexually mature female fathead minnows (*Pimephales promelas*). Linear programming was used to calculate optimal flux distributions required to maintain these system demands. Finally, network robustness was tested by sequentially deleting rank-ordered (low to high metric) reactions with effects on metabolite flux-sums, or average of flux consuming/producing a representative steroid metabolite, used as indicator of system robustness. *In silio* analyses are summarized in Supplemental 2.

2. Materials and methods

2.1. Ex vivo experimentation and mass spectrometric analysis

Methods detailing culture of adult (≥ 5 months old) female fathead minnows (*Pimephales promelas*) and steroid hormone quantification using liquid chromatography coupled with tandem mass spectrometry (LC-ESI+/MS/MS) are described elsewhere (Hala et al., 2011, 2012). For brevity, *ex vivo* experimentation comprised excising small segments of ovary tissue ($\sim$30–40 mg) from terminally sacrificed fathead minnows. Tissue segments were cultured for 24 h in a 25 °C water bath using buffered growth media (pH 7.5) supplemented with 1 μg/ml 25-hydroxycholesterol (0.04 μM) as cholesterol analog. 25-hydroxycholesterol uptake rate into female fathead ovary tissue has been previously quantified (Breen et al., 2007). Hereafter, cholesterol and 25-hydroxycholesterol are used inter-changeably as metabolism of either substrate to steroid hormones is stoichiometrically conserved. Prior to mass spectrometric analysis, culture media was spiked with deuterated internal standards (d_3-17α-hydroxypregnenolone, d_3-17β-estradiol and d_9-progesterone) and steroid hormones were extracted from media using liquid:liquid extraction with 1:1 $^{v}/_{v}$ hexane:ethyl acetate as solvent. Extracted steroids were derivatized and quantified using LC-ESI+/MS/MS. Steroid hormones quantified included: androgens (testosterone and 11-ketotestosterone), progestins (progesterone, pregnenolone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, and 17α,20β-dihydroxypregnenone), estrogens (17β-estradiol and estrone), and a corticosteroid (11-deoxycortisol) (Table 1).

2.2. Steroidogenesis model construction

The steroid model comprised a $m \times n$ stoichiometric matrix (A) of 'm' metabolites (rows) and 'n' reactions (columns). In total the model comprised 116 metabolites and 194 reactions (of which 74 were transport or exchange reactions) representing the mass balance (as stoichiometric coefficients) of metabolites consumed (negative integers) and produced (positive integers) in steroidogenic reactions. Reversible reactions were represented as two 'forward' reactions with the product metabolite from the first reaction acting as substrate in the second. All reactions were elementally balanced using cofactors (or 'currency' metabolites) such as oxygen (O_2), protons (H^+), carbon dioxide (CO_2), (reduced) nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH), and (reduced) nicotinamide adenine dinucleotide (NAD^+/NADH)

Table 1
List of cholesterol uptake and steroid metabolite production rates quantified from *ex vivo* culture of ovary tissues. All rates are presented as femtomole steroid hormone per milligram ovary tissue per hour or fmol/mg/h ($\pm$ standard error of mean).

Steroid hormones	Uptake(−) and production (+) rates (fmol/mg/hr)
25-hydroxycholesterol (cholesterol)	−39.83
Progesterone	0.02 ($\pm$0.003)
Pregnenolone	0.05 ($\pm$0.01)
17α-hydroxyprogesterone	0.05 ($\pm$0.02)
17α-hydroxypregnenolone	0.05 ($\pm$0.03)
17α,20β-dihydroxypregnenone	1.12 ($\pm$0.15)
17β-estradiol	0.20 ($\pm$0.06)
Estrone	0.08 ($\pm$0.02)
Testosterone	0.31 ($\pm$0.08)
11-ketotestosterone	0.03 ($\pm$0.01)
11-deoxycortisol	0.06 ($\pm$0.01)