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In silico predicted transcriptional regulatory control of steroidogenesis in spawning female fathead minnows (*Pimephales promelas*)

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ABSTRACT

Oocyte development and maturation (or oogenesis) in spawning female fish is mediated by interrelated transcriptional regulatory and steroidogenesis networks. This study integrates a transcriptional regulatory network (TRN) model of steroidogenic enzyme gene expressions with a flux balance analysis (FBA) model of steroidogenesis. The two models were functionally related. Output from the TRN model (as magnitude gene expression simulated using extreme pathway (ExPa) analysis) was used to re-constrain linear inequality bounds for reactions in the FBA model. This allowed TRN model predictions to impact the steroidogenesis FBA model. These two interrelated models were tested as follows: First, *in silico* targeted steroidogenic enzyme gene activations in the TRN model showed high co-regulation (67–83%) for genes involved with oocyte growth and development (cyp11a1, cyp17-17,20-lyase, 3β-HSD and cyp19a1a). Whereas, no or low co-regulation corresponded with genes concertedly involved with oocyte final maturation prior to spawning (cyp17-17α-hydroxylase (0%) and 20β-HSD (33%)). Analysis (using FBA) of accompanying steroidogenesis fluxes showed high overlap for enzymes involved with oocyte growth and development versus those involved with final maturation and spawning. Second, the TRN model was parameterized with *in vivo* changes in the presence/absence of transcription factors (TFs) during oogenesis in female fathead minnows (*Pimephales promelas*). Oogenesis stages studied included: PreVitellogenic-Vitellogenic, Vitellogenic-Mature, Mature-Ovulated and Ovulated-Atretic stages. Predictions of TRN genes active during oogenesis showed overall elevated expressions for most genes during early oocyte development (PreVitellogenic-Vitellogenic, Vitellogenic-Mature) and post-ovulation (Ovulated-Atretic). Whereas ovulation (Mature-Ovulated) showed highest expression for cyp17-17α-hydroxylase only. FBA showed steroid hormone productions to also follow trends concomitant with steroidogenic enzyme gene expressions. General trends predicted by *in silico* modeling were similar to those observed *in vivo*. The integrated computational framework presented was capable of mechanistically representing aspects of reproductive function in fish. This approach can be extended to study reproductive effects under exposure to adverse environmental or anthropogenic stressors.

1. Introduction

Within living systems the difficult task of multilevel integration, from genome to phenome, is accomplished using interrelated biological networks (Barabasi and Oltvai, 2004; Kitano, 2002). Perturbed phenotypes arise when the coordinated functions of such networks are disrupted by adverse genetic and/or environmental events (Hood et al., 2004; Schadt et al., 2009). Therefore given such complexity, there is great interest and need to identify the underlying organizing principles of networks and unify multilevel biological complexity (Frazier et al., 2003; Kitano, 2004; Mesarovic et al., 2004).

Towards this end, the applications of various graph-theoretic approaches have contributed considerably to the mathematical representation and study of complex biological networks. These approaches have provided deep insights into the fundamental connectivity properties of such networks (Barabasi and Oltvai, 2004; Jeong et al., 2000; Milo et al., 2002; Nacher and Akutsu, 2007; Wuchty et al., 2003) and illuminated conserved functions shaped by evolutionarily selective constraints (Barabasi and Albert, 1999; Ma and Zeng, 2003; Wagner and Fell, 2001). Despite such comprehensive descriptions, attempts to integrate multilevel models of disparate network sub-types (i.e. transcriptional regulatory and

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metabolic) have been limited. This is understandable given the challenge of unifying systems putatively operating under distinctive logical rules and/or functional constraints. While the functional logic of transcriptional regulatory networks readily lends to Boolean representation and interpretation (Albert and Othmer, 2003; Kauffman, 1969; Thomas, 1973); the mass-balance properties of metabolic networks is uniquely captured by differential equations or optimization approaches (Fell and Small, 1986; Majewski and Domach, 1990; Rapoport et al., 1976; Schauer et al., 1981; Teusink et al., 2000; Varma and Palsson, 1994). Therefore, a key challenge for constructing multilevel (and integrated) models is the *functional unification* of disparate networks in a manner that perturbation of one, cascades to and influences the other.

Thus far, various efforts have successfully integrated Boolean rules describing transcriptional activation as 'constraints' for metabolic network models. These approaches have been used to simulate prokaryote (*Escherichia coli*) growth under changing substrate and cofactor availabilities (Covert et al., 2001; Samal and Jain, 2008). Variations of such approaches have also been used to incorporate the signal transduction control of metabolic function (Covert et al., 2008; Papin and Palsson, 2004), and simulate interactions amongst multi-cell/tissue metabolic networks (Bordbar et al., 2011; Stolyar et al., 2007). A common 'kernel' amongst these approaches is the stoichiometric matrix representation of biological networks. As a mathematical construct, stoichiometric matrices encapsulate the connectivity architecture and information 'flow' of the system under study, and can be used to succinctly describe both transcriptional regulatory and metabolic networks (Gianchandani et al., 2006; Orman et al., 2011; Planes and Beasley, 2008). A stoichiometric matrix represents network components (transcription factors/metabolites) as mass-balanced participants in the system's representation. In this matrix, negative/positive coefficients are used to indicate consumption/production of transcription factors or metabolites through inter-related biological pathways. Such a unified representation allows the use of mathematical operators to 'solve' properties representative of system functions. For example, sub-space analysis (using extreme pathway or ExPa analysis) of transcriptional regulatory networks allows identifications of uniquely activated genes subject to the presence/absence of transcription factors (Gianchandani et al., 2009, 2006). And when applied to metabolic networks, ExPa can provide insights into structural (or topological) properties required to maintain steady-state metabolic functions (Hala and Huggett, 2014; Papin et al., 2002a, 2002b; Price et al., 2002; Schilling and Palsson, 1998; Wiback and Palsson, 2002). In addition, optimization approaches (such as flux balance analysis or FBA) can also be used to calculate optimal metabolic flux distributions (i.e. enzymes and associated catalytic capabilities) required to maximize stated network objectives, such as metabolite and/or biomass yields (Orth et al., 2010; Varma and Palsson, 1994). The calculation of optimal solutions is subject to the imposition of linear inequality constraints on the reactions comprising the model, therefore limiting both the *allowable* and *attainable* solutions of the system under study. Integration of genomic regulatory constraints as limits on (min/max) flux capabilities allows for functional integration between gene regulation and metabolism (Akesson et al., 2004; Colijn et al., 2009; Edwards and Palsson, 2000; Oberhardt et al., 2009).

This study uses an integrated *in silico* computational framework to investigate the transcriptional regulatory control of steroidogenesis during oocyte differentiation and maturation (or oogenesis) in spawning female fathead minnows (*Pimephales promelas*). Typically, oogenesis is organized into distinctive stages, including: pre-vitellogenic, vitellogenic, mature, ovulated and atretic stages (Tyler and Sumpter, 1996). Pre-vitellogenic – vitellogenic (PreVtg-Vtg) stages comprise mainly androgen- and estrogen-driven primary oocyte growth and sequestration of hepatically-derived proteins (such as pre-cursor to the egg yolk, vitellogenin) (Brooks et al., 1997). This stage is followed by oocyte maturation (Vtg-Mature) during which the oocyte prepares for ovulation by completing its first meiotic division (Nagahama and Yamashita, 2008). Oocyte maturation and ovulation (Mature-Ovulated) prior to spawning can be seen as a continuum induced by a surge in progestogen hormones (such as 17α,20β-dihydroxypregnenone). These hormones act as maturation inducing hormones, which work in concert with proteinase enzymes to stimulate egg release (Nagahama and Yamashita, 2008; Zhou et al., 2007). Following ovulation (Ovulated-Atretic), post-ovulatory follicles comprise remnants of steroid producing (granulosa/thecal) cells (Leino et al., 2005). Finally, oocytes that fail to ovulate are degraded via oocyte atresia (Tyler and Sumpter, 1996).

Each stage of oogenesis is accompanied with distinctive flux distributions in steroidogenesis (or steroid hormone productions), which are mediated by the concerted activities of cytochrome P450 (cyp450) and hydroxysteroid dehydrogenase (HSD) enzymes (Kime, 1993; Lubzens et al., 2010). Steroidogenesis is initiated upon cholesterol uptake into the mitochondria by the cholesterol transporter protein, steroidogenic acute regulatory protein (StAR). Imported cholesterol is subsequently converted to pregnenolone by the P450 side-chain cleavage enzyme (P450scc or cyp11a1) enzyme (Stocco and Clark, 1996), and released to the cytosol. Once released, pregnenolone is used as substrate by various cyp450 (cyp17, cyp19a1a) and HSD (3β-HSD, 20β-HSD) enzymes (localized on endoplasmic reticuli) to produce functionally relevant steroid hormones, such as androgens, estrogens, progestogens and corticoids (Payne and Hales, 2004). In turn, these hormones are key drivers for oocyte growth and maturation in spawning fish (Kime, 1993; Nagahama, 1994). The overall interplay between steroidogenesis and oogenesis is controlled by neuro-endocrine hormones (produced in pituitary) called gonadotropins. These neuro-endocrine hormones include follicle stimulating and luteinizing hormones (FSH and LH respectively) the productions of which are responsive to changes in environmental and/or metabolic cues, such as photoperiod, temperature, water quality and organismal energetic status (Billard et al., 1981; Kime 1993; Omura and Morohashi, 1995; Schulz et al., 2001; Chong et al., 2005).

In order to integrate and computationally model the multilevel complexity of oogenesis, i.e. from transcriptional regulation of steroidogenic enzyme genes to the control of steroidogenesis and associated hormone productions, an integrated *in silico* computational biology framework was implemented (Fig. 1). Existing approaches for studying piscine reproductive endocrine systems have used biochemical pathway maps to assist with mechanistic hypothesis generation (subject to perturbations) (Ankley et al., 2009; Villeneuve et al., 2007); or differential equations to relate changes in steroid hormone concentrations within an abbreviated network topology (Breen et al., 2007; Shoemaker et al., 2010). In contrast to these methods, the approach presented in this study uses stoichiometric matrices to represent both transcriptional regulatory and flux networks of steroidogenesis. For the first time, the two networks are functionally integrated whereby gene activation states predicted by the transcriptional regulatory network model, constrain the performance capabilities of the steroidogenesis flux model, influencing productions of steroid hormones.

Boolean rules describing transcriptional regulatory control of key steroidogenic genes, namely: StAR, cyp11a1, cyp17, cyp19a1a (aromatase), 3β-HSD and 20β-HSD, were represented as a pseudo-stoichiometric matrix model. This transcriptional regulatory network (TRN) model was constrained with the availability (presence/absence) of transcription factors (TFs). Subsequently, subspace (ExPa) analysis was used to predict the magnitude of gene activations subject to TF availability. In turn, gene expression lev-

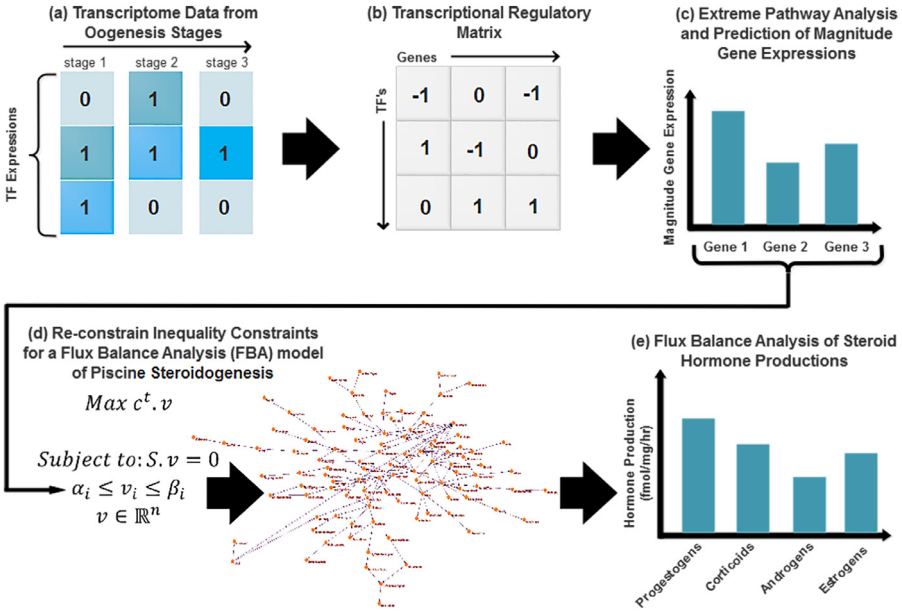

Fig. 1. Schematized summary of the *in silico* integrative computational framework used to study the transcriptional regulatory control of steroidogenesis during oogenesis in female fathead minnows. Transcription factor (TF) availability (presence/absence) was inferred during distinctive oogenesis stages from microarray data (NCBI GSE18254 dataset) (a), and used to parameterize a stoichiometric transcriptional regulatory network (TRN) model of steroidogenic enzyme gene regulation in ovary tissue (b). In turn, extreme pathway analysis was used to predict the magnitude expression changes for steroidogenic enzyme genes (c). Expression levels were used to re-constrain inequality bounds for a flux balance analysis (FBA) model of piscine steroidogenesis (d), and predict changes in steroid hormone productions during oogenesis (e).

els were used to re-constrain the catalytic capabilities for transcribed enzymes participating in a stoichiometric model of piscine steroidogenesis (Fig. 1) (Hala and Huggett, 2014; Hala et al., 2015). Once constrained, FBA was used to predict steroid hormone productions. *In silico* TRN and FBA predictions were firstly tested under *in silico* targeted gene activations and then with *in vivo* experimentally derived data. Specifically, microarray data generated by Villeneuve et al. (2010) quantifying gene expression changes during oogenesis in female fathead minnows (*Pimephales promelas*) (NCBI GSE18254 dataset) were used to constrain the TRN model. Subsequently, TRN model predictions of magnitude gene activations for steroidogenic enzyme genes were used to re-constrain reaction catalytic capabilities in the steroidogenesis FBA model, allowing context-specific predictions of steroid hormone productions during oogenesis. Finally, model predictions were compared and contrasted with published knowledge of steroidogenic enzyme gene expressions and steroid hormone productions in spawning female fathead minnows.

2. Materials and methods

2.1. Transcriptional regulatory network (TRN) construction and extreme pathway (ExPa) analysis

Boolean rules describing transcription factor (TF) control of key steroidogenic enzyme genes (Table 1) were mapped to a pseudo-stoichiometric matrix model using formalisms described by Gianchandani et al. (2006, 2009) (**Supplemental 1: TRN Matrix**). Boolean rules were derived after a thorough review of the literature and represent a totality of current knowledge on the TF mediated regulation of vertebrate steroidogenic enzyme genes (Table 1). The matrix mathematically represented (as 'mass-balances') TFs participating in gene regulation as negative integers, whereas activated genes, inactivated genes and associated transcript productions were represented as positive integers. The model comprised seventeen TF's, which mainly constituted small peptide/protein factors or activated receptors. Initially, six key steroidogenic genes were annotated, including StAR, cyp11a1, cyp17 and cyp19a1a, 3β-HSD and 20β-HSD. Eventually, cyp17 was annotated as two separate genes (17,20-lyase or 17α-hydroxylase) since its transcript enzyme exhibits two catalytically distinctive activities, with each controlling unique steroidogenic flux distributions (Conley and Bird, 1997; Halm et al., 2003; Sakai et al., 1992). Cyp17-17,20-lyase catalyzes conversion of progestogens (17α-hydroxypregnenolone, 17α-hydroxyprogesterone) to androgens (dehydroepiandrosterone, androstenedione) via cleavage of carbon17-20 bond on progestogens (**Supplemental 2**). Alternatively, cyp17-17α-hydroxylase activity catalyzes 17α-hydroxylation of carbon17 on progestogens (pregnenolone and progesterone) to produce 17α-hydroxypregnenolone and 17α-hydroxyprogesterone (which is subsequently catalyzed to the oocyte maturation inducing hormone, 17α,20β-dihydroxypregnenone via 20β-HSD) (**Supplemental 2**). Each catalytic state is dependent upon gonadotropin (FSH) mediated phosphorylation of cyp17, with phosphorylated cyp17 mediating 17,20-lyase activity and de-phosphorylation yielding 17α-hydroxylase activity (Nagahama, 1994; Nagahama and Yamashita, 2008; Sreenivasulu and Senthilkumaran, 2009). Therefore, each catalytic state of cyp17 (17,20-lyase or 17α-hydroxylase) was distinctly annotated as two separate 'genes' (each with its own distinctive TF regulation) in the *in silico* TRN model. The inclusion of these two catalytic states for cyp17 yielded a final count of seven genes, regulated by seventeen TFs (Table 1).

The final TRN model accounted for all activation (AC) or inactivation (IN) states for each gene as dictated by Boolean AND, OR, NOT formalisms in the column vector of the TRN model. The AND formalism indicates that the coordinated presence of all named TFs are required to activate a particular gene; OR indicates that the presence of either TF is sufficient to activate the gene; whereas NOT indicates that the absence of specific TF's are needed for the gene to be active (i.e. these TFs have an inhibitory effect on gene expression). This vector was also augmented with an 'exchange' (EX) matrix that indicated the output of peptides transcribed by steroidogenic enzyme genes out of the model (**Supplemental 1: TRN Matrix**). Furthermore, TF and transcript peptide

Table 1
List of Boolean rules describing the regulation of key ovarian steroidogenic enzyme genes by seventeen transcription factors (TFs). (Boolean formalism key: and = all TFs required for gene activation; or = either TF required for gene activation; not = absence of TFs required for gene activation) (TF key: LH = luteinizing hormone; LHR = luteinizing hormone receptor; FSH = follicle stimulating hormone; FSHR = follicle stimulating hormone receptor; SCAP = sterol regulatory element-binding protein (SREBP) cleavage-activating protein; INSIG = insulin-induced gene; DAX-1 = dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; Ad4BP/SF-1 = adrenal 4 binding protein/steroidogenic factor 1; IGF-1 = insulin-like growth factor 1; EGF = epidermal growth factor; PGF2α = prostaglandin F2α; TNFα = tumor necrosis factor α; IL-1 = interleukin-1; BMP-2 = bone morphogenic protein 2; DMRT1 = doublesex and mab-3 related transcription factor 1; AMH = anti-müllerian hormone).

Genes	Boolean Rules	References
StAR	(LH and LHR) or (FSH and FSHR) or (not(SCAP and INSIG)) or (not(DAX-1))	Lavoie and King (2009), Sandhoff and McLean (1999), Stocco (2001), Ye and DeBose-Boyd (2011)
Cyp11a1	(LH and LHR) or (FSH and FSHR) or (Ad4BP/SF-1) or (IGF-1)	Guo et al. (2003), King and LaVoie (2012), Winters et al., (1998)
Cyp17-17,20-lyase	(FSH and FSHR) or (cytochromeB5)	Conley and Bird (1997), Nagahama and Yamashita (2008)
Cyp17-17α-hydroxylase	(not(FSH and FSHR) or (cytochromeB5)	Conley and Bird (1997), Nagahama and Yamashita (2008)
3β-HSD	(FSH and FSHR) or (EGF) or (IGF-1)) and (not(PGF2α and TNFα and IL-1 and BMP-2))	Lavoie and King (2009)
Cyp19a1a	((FSH and FSHR) or (Ad4BP/SF-1) or (IGF-1)) and (not(DMRT1 and AMH))	Leet et al. (2011), Rodriguez-Mari et al. (2005)
20β-HSD	(LH and LHR)	Nagahama and Yamashita (2008), Senthilkumaran et al. (2004)

presence/absence was also represented as the row vector of the TRN model (Gianchandani et al., 2009, 2006). Therefore, the TRN matrix was represented as a 48×63 $m x n$ dimension matrix. Finally this matrix was also augmented with an incidence 'environment' matrix that allowed inclusion of condition-specific TF presence/absence or availability (AV) as determined from experimentation. Once defined in this manner, the entire TRN model comprised a 48×80 $m x n$ dimension matrix (**Supplemental 1: TRN Matrix**). This matrix was solved for convex null space vectors using the ExPa algorithm developed by Bell and Palsson (2005). ExPa calculated sets of non-negative basis vectors (or extreme pathways) spanning the null space of the TRN matrix. These vectors are comprised of linearly-independent variables (or genes) that balance the steady state conditionality of the TRN matrix. Therefore, the computed sets of vectors indicated the activation states of genes subject to the context-specific (and experimentally determined) presence/absence of TFs (Schilling and Palsson, 1998). In turn, the 'magnitude' of expression for any particular active gene (m_g) was calculated as follows:

$$m_g = \frac{Number\ of\ times\ g\ \neq 0\ in\ ExPa\ basis\ vectors}{Number\ of\ attainable\ activation\ states\ for\ g}$$

i.e. by normalizing the total *representation* (as integer indices >0) of each gene (g) in the vectors generated during ExPa analysis relative to the sum total numbers of gene activation states *attainable* by Boolean AND, OR operators. This analysis yielded a representative 'pseudo-expression' level for each gene as fully activated genes were subject to the presence of all activating TFs. In turn, the magnitude of expression level for each steroidogenic enzyme gene (as represented by mg) was used to determine min/max constraints for transcribed enzymes and associated reactions participant in the stoichiometric flux balance analysis (FBA) model of steroidogenesis (Fig. 1).

2.2. In silico steroidogenesis and flux balance analysis (FBA)

A previously developed stoichiometric model of piscine steroidogenesis was used for all simulations (Hala and Huggett, 2014; Hala et al., 2015). For brevity, the model mapped mass-balanced transformations of 118 metabolites in 124 inter-related steroidogenic enzyme catalyzed reactions (**Supplemental 1: Reactions, S-Matrix and Gene Rules**). The stoichiometric matrix was also augmented with 75 exchange reactions allowing metabolite or cofactor transport into/out of the model. These exchange reactions also represented productions (as output from the model) of 55 steroid hormones. Metabolites moving into the model were represented with positive coefficients, whereas those leaving were represented by negative coefficients. Therefore, the overall model comprised a $m x n$ dimension stoichiometric matrix of 118 metabolites and 199 reactions (**Supplemental 1: S-Matrix and Gene Rules**). Seventy percent of reactions in the model were annotated with Entrez Gene I.D.'s for respective steroidogenic enzyme genes. The cholesterol import reaction (EX_M1) mediated by StAR was also annotated. In total 39 genes in various Boolean configurations were used to annotate 88 reactions (including the cholesterol import exchange reaction).

The overall steroidogenic model was framed in vector form as a linear programming optimization problem (Orth et al., 2010; Savinell and Palsson, 1992):

$$Max\ c^t.v$$

$$Subject\ to: S.v = 0$$

$$\alpha_i \leq v_i \leq \beta_i$$

$$v \in \mathbb{R}^n,\ n = 199$$

The objective function ($\max c^t.v$) constituted the inner product of the transposed $1xn$ cost vector (c^t) and $nx1$ vector of network reactions. The vector c^t was set to a specific (biomass) exchange reaction that consumed key steroidogenic intermediate metabolites to produce per unit gonad biomass (Hala et al., 2015). This pseudo-reaction represented contributions of key steroid hormones for gonad growth (Bhatta et al., 2012; Jensen et al., 2001; Yaron and Levavi-Sivan, 2011). Therefore, all entries of c^t were null (0) except for the biomass exchange reaction (which was set to 1). Optimal values for network reactions ($v \in \mathbb{R}^n$) were solved using flux balance analysis (FBA) as initialized in the COBRA toolbox (v3.0) and solved using the GNU linear programming kit (glpk) solver in MATLAB^{TM} (R2015a) (Becker et al., 2007; Schellenberger et al., 2011). The optimal values computed represent the catalytic capabilities of steroidogenic reactions (or flux distributions) that maximize the biomass objective function while satisfying the imposed linear inequality constraints. An optimal flux distribution is calculated by framing the 118×199 $m x n$ dimension steroidogenic stoichiometric matrix model as a product at steady state (i.e. $S.v = 0$). All reactions in the model were constrained with min/max bounds ($\alpha_i \leq v_i \leq \beta_i$) with a null lower bound ($\alpha_i = 0$) and arbitrarily large upper bound of 1000 fmol/mg/hr ($\beta_i = 1000$) for all enzyme catalyzed reactions. Exchange reactions for 'currency metabolites' (such as co-factors, protons and oxygen) were set $-1000/1000$ α_i/β_i, with only the cholesterol import (EX_M1) reaction set to the experimentally determined rate of 40 fmol/mg/hr

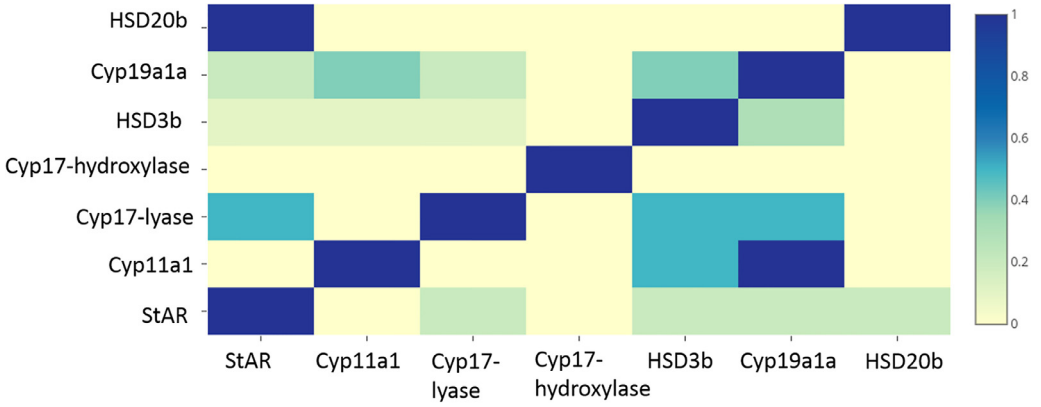

Fig. 2. Heat-map showing extreme pathway analysis predictions of gene co-regulations during *in silico* sequential and targeted gene activations of the seven steroidogenic enzyme genes constituting the transcriptional regulatory network (TRN) model. The minor diagonal (lower left to upper right corner) shows the order of activation for genes and co-regulated genes are shown off diagonal. The scale represents non-active (light color) to fully activated genes (darkest color).

into ovarian tissue of female fathead minnows (*Pimephales promelas*) (Breen et al., 2007) (**Supplemental 1: S-Matrix Constraints**).

Finally, context-specific magnitude gene expression changes for steroidogenic enzyme genes (in the TRN model) as computed using ExPa analysis were used to constrain min/max reaction bounds for transcribed enzymes in the steroidogenesis model. For inactive genes, associated enzyme min/max constraints were set to 1% of max (i.e. −10/+10 instead of default -1000/+1000). This represented a tight or limiting penalty on constraints and prevented a null or zero flux constraint being imposed for reactions transcribed by non-expressed genes. A null constraint can lead to a blocked flux distribution, dramatically limiting the computability of a solution. The seven steroidogenic enzyme genes modeled as TRNs mapped to 26% of enzyme catalyzed reactions in the steroidogenic model (including the cholesterol import protein, StAR). The influence of gene activations on steroidogenic flux distributions was tested under targeted *in silico* or experimentally derived *in vivo* gene-activations.

2.3. In silico targeted enzyme gene activations and associated steroidogenic fluxes

The ability of the TRN model to accurately predict targeted gene activations was tested. This was done to confirm TRN predictions of the magnitude of gene activation as a 100% expression for each targeted gene was expected. As the Boolean rules for each gene codified the conditional presence of TFs required to activate that gene, TFs were (sequentially) made available for each gene and extreme pathway basis vectors calculated. For each gene, a 100% activation was achieved, confirming that the TRN model was able to accurately predict gene activations subject to the full presence of TFs required to activate any particular gene. In addition, as shared TF usage was evident in the Boolean rules for each gene, the ancillary activation of a target gene also led to co-activations of additional (co-regulated) genes (Fig. 2).

The subsequent impact of *in silico* targeted gene activations on steroidogenic flux distributions was studied using flux balance analysis (FBA). For enzymes transcribed by isozyme (AND) or isoform (OR) Boolean operators, expression values were either averaged (isozyme complexes) or a uniformly random expression level was chosen (for isoform complexes) from a range spanning [10, 1000]. The expression level of each gene was used as a scalar to re-constrain the upper and/or lower bounds for transcribed enzymes in the steroidogenesis model (Colijn et al., 2009). Therefore, if a gene was maximally expressed (i.e. magnitude expression = 1.0 or 100% active), its associated max (β_i) flux constraint would remain unchanged (i.e. 1000 * 1.0 = 1000.0). Likewise, a lower magnitude expression (e.g. 0.5 or 50% active) would halve the associated flux constraint (i.e. 1000 * 0.5 = 500.0). For non-expressed genes a default flux constraint 1% of max was chosen (i.e. min/max = -10/10). This rule prevented a null min/max flux constraint for a reaction. The resultant system with re-scaled min/max constraints was solved as a linear programming optimization problem using FBA. Relationships amongst resultant flux values and transcribed enzymes were analyzed using correspondence analysis (CA) (Fig. 3(a)).

2.4. TRNs and steroidogenesis during oogenesis in female fathead minnows

Expression levels for the TFs represented in the TRN model were parsed from 15,000 gene microarray datasets (GEO, GSE18254) quantified during five distinctive oogenesis stages in female fathead minnows: pre-vitellogenic, vitellogenic, mature, ovulated and atretic oocytes (Villeneuve et al., 2010). Four replicate array datasets per oogenesis stage were parsed for expression levels of TFs. An exact match was found for 59% of TFs in the TRN model relative to genes in the microarrays (i.e. only 10 out of the 17 TFs represented in the TRN model were exactly matched with genes in the microarrays). For the TF genes with no representation in microarrays, their representation in the TRN model was set to absent (0). All expression levels were $\log_{10}$ transformed and transient changes between oogenesis developmental stages (PreVtg-Vtg, Vtg-Mature, Mature-Ovulated, Ovulated-Atretic) were used to infer TF presence/absence. Transient stages included: Pre-Vitellogenic – Vitellogenic (PreVtg-Vtg), Vitellogenic – Mature (Vtg-Mature), Mature – Ovulated and Ovulated - Atretic. A 20% threshold was used as cut-off to infer expression changes (i.e. if the difference between two developmental stages was ≥20% of the initial value, then the TF was set to unavailable (0); or if the difference was <20% the initial value, then it was set to available (1)). In such a manner, the augmented environment matrix of the TRN model was parameterized for the presence/absence of TFs (as described in Section 2.1). A more stringent threshold of 50% was also tested and the 20% threshold criteria was found to be in 88–91% agreement (across the four oogenesis stages) for predictions of TF availability/unavailability. Therefore, a higher threshold selection was not expected to affect computational analysis. ExPa analysis was used to calculate the context-specific (i.e. based on oogenesis stage) steroidogenic enzyme gene activation states. As described previously (Section 2.3), ExPa predicted magnitude gene activations were used as constraints for FBA. Taken together, ExPa

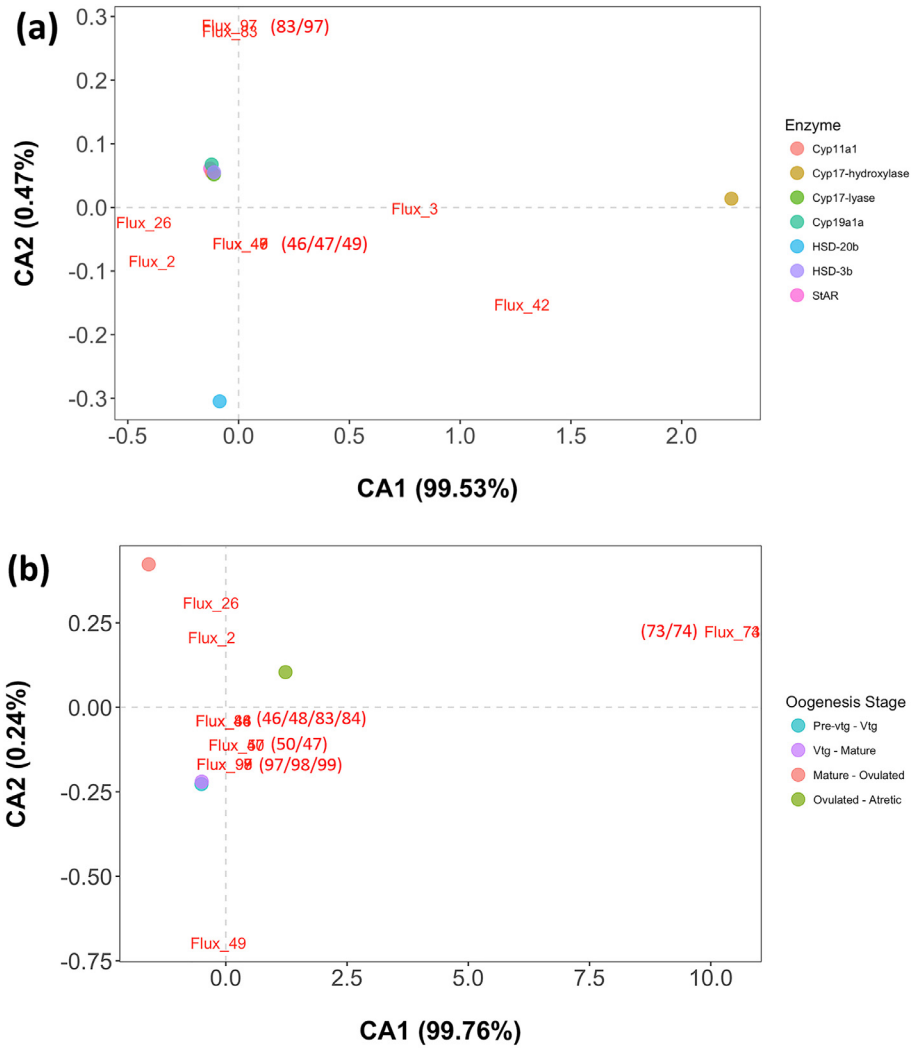

Fig. 3. Correspondence Analysis (CA) biplots showing ordinations and associated fluxes responsible for influencing their distributions for: (a) *in silico* targeted key steroidogenic enzyme gene activations in the transcriptional regulatory network (TRN) model, and (b) analysis of distinctive oogenesis stages in female fathead minnows (*Pimephales promelas*) (Please consult **Supplemental 1: Enzyme-CA Flux Contribution** and **Oogenesis-CA Flux Contribution** for information on reactions and catalyzed products).

and FBA allowed exploration of changes in steroidogenic enzyme gene expression changes and associated steroidogenesis flux distributions during oogenesis. Relationships amongst flux values and oogenesis stages were analyzed using correspondence analysis (CA) (Fig. 3(b)).

2.5. Correspondence analysis

Correspondence analysis (CA) was performed to explore relationships amongst targeted gene activations or oogenesis developmental stages and associated flux values. Flux values for reactions consistently conserved amongst FBA solution vectors (from *in silico* targeted gene activations or *in vivo* experimentation) were Log_{10} transformed to normalize large magnitude variations. CA was conducted using the *vegan* package (Oksanen et al., 2017) in the R statistical program (v3.3.3) with relationships amongst covariates visualized as a biplot of the first two CA axes.

3. Results and discussion

3.1. Formulation of Boolean regulatory rules

Boolean rules as shown in Table 1 were used to concisely state the conditional logic of TF presence/absence as required to regulate steroidogenic enzyme gene expressions. These rules were derived from primary literature and are cited as appropriate. As can be seen in Table 1, a key controller of all genes are the gonadotropin hormones (FSH and LH) along with associated membrane receptors (FSHR and LHR) (Yaron et al., 2003). These glycoprotein hormones are produced in the anterior pituitary under stimulation from the hypothalamic neurohormone, gonadotropin releasing hormone (or GnRH). Elevated FSH and LH secretion corresponds with elevated activities of the mitochondrial cholesterol transporter (StAR) (Lavoie and King, 2009; Stocco, 2001). Whereas the sterol regulatory element-binding protein (SREBP) and cleavage-activating protein (SCAP) complex acts as a cholesterol sensor that simulates lipid synthesis and cholesterol production, therefore increasing the levels of a key substrate (cholesterol) for StAR import (Shimizu-Albergine et al., 2016). In turn, StAR activity is inhibited by insulin-induced gene-1/2 (INSIG) activity which prevents SCAP/SREBP complex release (from endoplasmic reticula and golgi apparatus) (Ye and DeBose-Boyd, 2011). In addition, DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X-chromosome, gene 1), the so called 'anti-testis' gene can also inhibit StAR, causing steroidogenic disruption (Goodfellow and Camerino, 1999; Sandhoff and McLean, 1999).

Cyp11a1 controls a key first step is steroid biosynthesis by converting cholesterol to pregnenolone (**Supplemental 2**). As with all steroidogenic enzyme genes, its expression is also controlled by gonadotropin (FSH and LH) binding to cognate G protein-coupled receptors and activations of downstream cAMP/PKA signaling pathways. In turn, these signaling pathways culminate with the assembly of transcriptional initiation complexes on the cyp11a1 promoter, which include steroidogenic factor-1/Ad4-binding protein (SF-1/Ad4BP) (Guo et al., 2003). Insulin-like growth factor 1 (IGF-1) can also stimulate cyp11a1 expression when administered alone or concomitantly with FSH (Winters et al., 1998) (Table 1).

As previously described in Section 2.1, the Boolean control of cyp17 expression was encoded into two separate catalytic activities, 17α-hydroxylase or 17,20-lyase (Table 1) (Nakajin and Hall, 1981). With each activity mainly controlled by gonadotropin (FSH) mediated de-phosphorylation (17α-hydroxylase activity) or phosphorylation (17,20-lyase activity) of the cyp17 enzyme (Conley and Bird, 1997; Nagahama and Yamashita, 2008; Zhang et al., 1995). An additional factor responsible for stimulating 17,20-lyase activity includes a hemeprotein (cytochrome b5) that acts as a constituent of microsomal oxidoreductase protein complexes (Huang et al., 2008). Studies with porcine testicular microsomal fractions demonstrate the dependence of cyp17-17,20-lyase activity on the presence of cytochrome b5 (Katagiri et al., 1982); with recent NMR analyses confirming cyp17/cytochrome b5 complex formation as being responsible for enhancing 17,20-lyase activity (Estrada et al., 2013). Given such clear conditional logic for the activation of cyp17-17,20-lyase activity, converse rules were taken to denote cyp17-17α-hydroxylase activation (Table 1).

Just as for cyp17, 3β-HSD activity is also under exquisite regulation by various TFs. IGF-I is a strong regulator of 3β-HSD in rat granulosa cells (not requiring presence of FSH) (Eimerl and Orly, 2002), whereas prostaglandins (PGF2α), tumor necrosis factor-α (TNFα) and bone morphogenic proteins (BMP-2) are potent suppressors of 3β-HSD expression and activity (Brankin et al., 2005; Lavoie and King, 2009; Li et al., 1993; Xiong and Hales, 1997) (Table 1). Finally, both cyp19a1a and 20β-HSD gene expressions are tightly coordinated by the neuroendocrine gonadotropin hormones, FSH and LH. While cyp19a1a expression is stimulated under FSH secretion, 20β-HSD expression is stimulated under LH secretion (Senthilkumaran et al., 2004). Furthermore, cyp19a1a is also actively repressed by somatic and germ cell factors (such as DMRT1 and AMH) during male-dependent sex determination and development in embryo-larval or juvenile life stages (Leet et al., 2011; Rodriguez-Mari et al., 2005) (Table 1).

3.2. Targeted in silico steroidogenic enzyme gene activations and impacts on steroidogenesis

The targeted activation of each steroidogenic enzyme gene in the gonad TRN model (subject to presence of all conditionally-required TFs) showed 100% expression of each gene (Fig. 2). Shared TF usage amongst genes resulted in co-activations (to varying extents) of additional genes (Fig. 2). Targeted activation of StAR and cyp11a1 showed the greatest extent of gene co-regulation by the TFs (83% for each), followed by cyp17-17,20-lyase, 3β-HSD and cyp19a1a (67% for each). These groups of genes tend to be functionally correlated during oocyte growth and development (just prior to final gamete maturation and spawning) (Kumar et al., 2000). Finally, 20β-HSD (33%) and cyp17-17α-hydroxylase (0%) showed lowest or no co-regulation respectively. The observed co-regulatory activity is representative of Boolean parameterization (Table 1), and correctly maps TF promiscuity encoded within each Boolean rule. Cyp17-17α-hydroxylase and 20β-HSD exhibited the lowest co-regulatory activities as these two enzymes are distinctly regulated during final gamete maturation. While increased cyp17-17α-hydroxylase activity corresponds with lowered FSH release; 20β-HSD activation is concomitant with an LH surge (Nagahama and Yamashita, 2008; Senthilkumaran et al., 2004; Sreenivasulu and Senthilkumaran, 2009). Therefore, StAR, cyp11a1, cyp17-17,20-lyase, 3β-HSD and cyp19a1a appear to comprise a set of (often) co-regulated genes that are likely to be functionally related.

Such co-regulatory behavior of steroidogenic enzyme genes also mapped to steroidogenesis fluxes. The correspondence analysis (CA) of Log_{10} transformed flux values showed highly overlapped ordinations for cyp11a1, cyp17-17,20-lyase, cyp19a1a, 3β-HSD and StAR near the origin (Fig. 3(a)). This indicated relatively homologous variances of fluxes for reactions 2, 26, 46, 47 and 49 (producing progestogens and an androgen), which together explained 29% of variance along the first CA axis (**Supplemental 1: Enzyme-CA Flux Contribution**). In contrast, ordinations for cyp17-17α-hydroxylase and 20β-HSD were dispersed across the first and second CA axes respectively (Fig. 3(a)). While cholesterol (reaction 3) and progestogen (reaction 42) metabolisms contribute towards explaining the variance of cyp17-17α-hydroxylase, androgen metabolism (reactions 83 and 97) was inversely related 20β-HSD (Fig. 3(a)). As discussed in Section 3.1, cyp17-17α-hydroxylase and 20β-HSD enzymes are differentially modulated during oocyte development and final maturation prior to spawning (i.e. being highly reliant on the juxtaposition of low FSH secretion and high LH surge) (Nagahama and Yamashita, 2008; Senthilkumaran et al., 2004; Sreenivasulu and Senthilkumaran, 2009). Therefore, the CA analysis alludes to an innate ability of differential gene co-regulations to also impact steroidogenic flux distributions.

3.3. In silico predicted steroidogenic TRNs activated during oogenesis

TRN analysis of steroidogenic enzyme gene activations during oogenesis in female fathead minnows showed the presence of distinctive regulatory states (Fig. 4). Of the seven steroidogenic genes, StAR, 3β-HSD, cyp19a1a and cyp17-17,20-lyase were constitutively active across all oogenesis stages, suggesting continued investment in steroid hormone productions during oogenesis. In contrast, 20β-HSD, cyp17-17α-hydroxylase and cyp11a1 show distinctive activations at specific oogenesis stages only. Early oocyte development (PreVtg-Vtg stages) indicated activations of a majority of steroidogenic enzymes, with exception of cyp17-17α-hydroxylase. This is consistent with the expectation that early oocyte development is mainly driven by androgen and estrogen productions, with key proponent enzyme systems constituting cyp11a1, cyp17-17,20-lyase, 3β-HSD and cyp19a1a. Cyp19a1a catalyzes conversions of androgens to estrogens and is prominently active during PreVtg-Vtg, Vtg-Mature and Ovulated-Atretic stages (subsequent to spawning). The elevated expressions for cyp19a1a are also concomitant with vitellogenin sequestration during early oogenesis.

Overall, steroidogenic enzyme gene activations decreased as oogenesis approached the Mature-Ovulated stage, with 20β-HSD and cyp11a1 not being expressed (Fig. 4). The absence 20β-HSD expression during the Mature-Ovulated stage is unexpected as it catalyzes production of the progestogen (and maturation-inducing hormone), 17α,20β-dihydroxypregnenone. This hormone is responsible for oocyte maturation prior to ovulation (Nagahama and Yamashita, 2008). However, reappearance of 20β-HSD expression at the Ovulated-Atretic stage indicates delayed (or just prior) involvement at ovulation, making its induction more prevalent at the Ovulated-Atretic stage instead. Complete cessation of cyp11a1 expression at the Mature-Ovulated stage appears unlikely as it catalyzes the first steroidogenic reaction (cholesterol $\rightarrow$ pregnenolone). As a result, cyp11a1 is an important proponent of steroidogenesis during development and reproduction in fish (Hsu et al., 2009, 2006). It is likely that down-regulation of cyp11a1 is indicative of overall downregulation for a majority

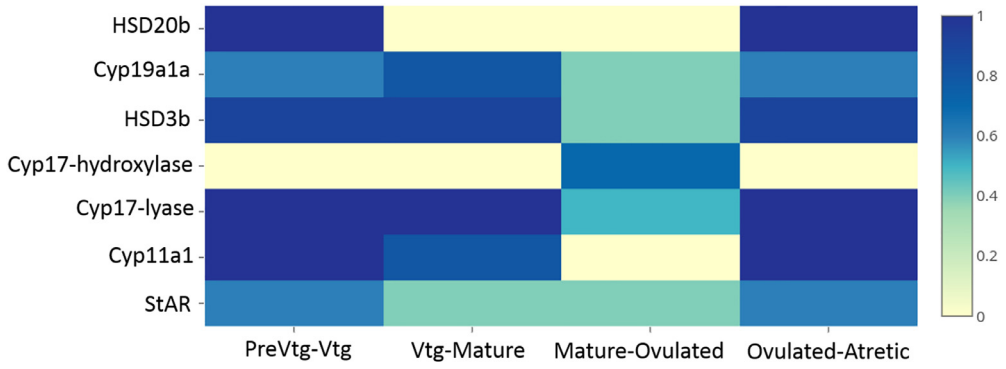

Fig. 4. Heat-map showing extreme pathway analysis predictions of steroidogenic enzyme gene activations during oogenesis in female fathead minnows (*Pimephales promelas*). Oogenesis stages shown include: PreVitellogenic-Vitellogenic (PreVtg-Vtg), Vitellogenic-Mature (Vtg-Mature), Mature-Ovulated and Ovulated-Atretic. The magnitude gene expression for each gene are averaged for an n = 4 females/oogenesis stage.

of steroidogenic enzyme genes at the Mature-Ovulated stage. It is likely that a sufficiently large down-regulation in cyp11a1 expression is captured as non-expressed in model predictions. In contrast, cyp17-17α-hydroxylase showed elevated expression for the Mature-Ovulated stage only. Elevated cyp17-17α-hydroxylase at the Mature-Ovulated stage and accompanying increase in 20β-HSD expression at the Ovulated-Atretic stage strongly implicates a distinctive shift in steroid hormones from androgens and estrogens to progestogens. This result aligns well with the role of progestogens as maturation inducing factors prior to oocyte ovulation (Nagahama and Yamashita, 2008; Senthilkumaran et al., 2004).

Fathead minnows exhibit an asynchronous (or fractional) spawning behavior, with a relatively high spawning interval of every 3–4 days (Jensen et al., 2001). As a result, all oocyte developmental stages are evident to varying extents during a reproductive cycle (Jensen et al., 2001; Leino et al., 2005), and highly contrasted steroidogenic gene expressions and associated steroid hormone productions are not expected. However, transcriptomics analyses (using 15,000 gene microarrays) of oogenesis in female fathead minnows does show evidence of contrasted transcriptomes between pre-vitellogenic and vitellogenic stages of development (Villeneuve et al., 2010). The same study also performed targeted quantitative PCR analysis for a majority of steroidogenic genes modeled in this study. Such targeted analyses revealed a general trend of elevated StAR, cyp11a1, cyp19a1a, 3β-HSD and 20β-HSD gene expressions during early oogenesis (pre-vitellogenesis), relative to later oogenesis developmental stages (Villeneuve et al., 2010). This trend is in overall agreement with *in silico* simulations presented in this manuscript. An exception however is noted with a return to higher gene expressions at the final Ovulated-Atretic stage *in silico* (which was not observed *in vivo* by Villeneuve et al., 2010). A reason for lack of agreement at this late developmental stage is not immediately clear. Differing stabilities of steroidogenic mRNAs under gonadotropin stimulations may be likely. For example, FSH stimulation has been shown to increase cyp19 mRNA half-life from 3 to 12 hours in bovine granulosa cells (Sahmi et al., 2006). This observation agrees well with studies showing that elevated transcript levels for FSH and LH correlate positively with those for steroidogenic enzyme genes in reproducing male and female fish (Kumar et al., 2000; Kusakabe et al., 2006). Such association between gonadotropin presence and steroidogenic enzyme gene activations is encoded with the Boolean rules used for TRN simulations. Furthermore, analysis of Villeneuve et al. (2010) transcriptomics datasets shows both FSH and LH to be concomitantly elevated at the PreVtg-Vtg and Ovulated-Atretic stages (data not shown), therefore making a mismatch between *in silico* predictions and *in vivo* observations difficult to explain. As *In silico* predictions are driven by transcription factor (TF) presence/absence, it is likely that TF levels are 'ramping-up' at the Ovulated-Atretic stage to prepare for a reset (or re-initialization) of oogenesis back to the PreVtg-Vtg stage. Therefore mismatch between *in silico* prediction vs. *in vivo* experimentation at the Ovulated-Atretic stage maybe due to post-translational delays through as-yet understudied mechanisms. In time, the discovery and inclusion of such mechanisms into Boolean rules will increase model resolution (as long as they are experimentally quantifiable).

3.4. In silico predicted steroidogenic fluxes and steroid hormone productions during oogenesis

The extent to which the magnitude of steroidogenic enzyme gene activations impacted steroid hormone productions during oogenesis was investigated using a stoichiometric flux balance analysis (FBA) model of piscine steroidogenesis. TRN predicted gene activation changes were used to re-scale the linear constraints for representative enzymes comprising the FBA model (Fig. 1). By proxy, such re-scaling served to limit the allowable and attainable fluxes across the steroidogenic network. The production of major classes of hormones was simulated, including progestogens (comprising 14 hormones), corticoids (8 hormones), androgens (19 hormones) and estrogens (14 hormones). This analysis showed steroid hormone production rates (as fmol/mg/hr) to mirror trends predicted for steroidogenic gene expression changes during oogenesis (Fig. 5). An overall oscillation was also seen for steroid hormone productions, with overall higher titers during early (PreVtg-Vtg) and late (Ovulated-Atretic) oogenesis, and lower titers during intermediate stages (Vtg-Mature and Mature-Ovulated) (Fig. 5).

Nuances in steroid hormone productions are largely dependent on the reproductive strategy employed by fish. In synchronously (i.e. annual or seasonal) spawning fish, plasma gonadotropin (FSH) and sex steroid hormone (androgens/estrogens) levels dominate during early oocyte development (i.e. encompassing PreVtg-Vtg stages). This is followed by an LH surge and predominance of progestogen production during oocyte maturation and ovulation (Rinchard et al., 1993; Scott and Canario, 1992; Sumpter and Scott, 1989). Few studies have investigated the correspondence between steroidogenic enzyme gene expressions and associated steroid hormone productions in synchronously spawning fish. However, Kumar et al. (2000) have shown an overall positive correlation between enzyme gene expressions for cyp11a1, cyp17, 3β-HSD and cyp19a1a, and circulating testosterone and estradiol levels over a seasonal spawning cycle in the channel catfish (*Ictalurus punctatus*).

In contrast, asynchronous spawners (such as fathead minnows) are not expected to exhibit distinctive shifts in hormone productions during spawning. Overall there is a paucity of information

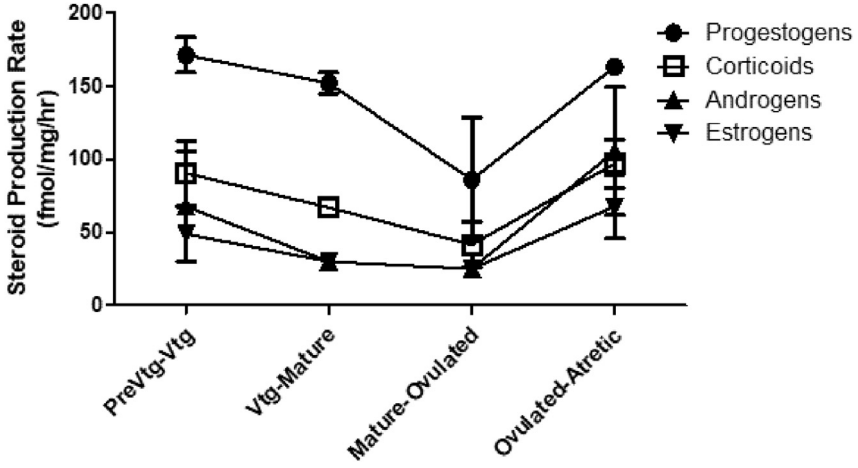

Fig. 5. Flux balance analysis (FBA) predictions of steroid hormone productions during oogenesis in female fathead minnows (*Pimephales promelas*). Values are shown as mean ± standard error (n = 4 females per stage).

on changes in steroid titers during a spawning cycle of fathead minnows. An insightful study by Jensen et al. (2001) showed cycling for plasma levels of an estrogen (17β-estradiol) and androgen (testosterone) in female fathead minnows during reproduction. Interestingly, levels of 17β-estradiol and testosterone were shown to increase (statistically significantly for the estrogen) 24 hours after spawning, and to steadily decline thereafter until the next spawning event. Given that immediately following spawning, predominant oogenesis stages comprise pre-vitellogenic and vitellogenic oocytes (Villeneuve et al., 2010), the concordant increase and then decrease for 17β-estradiol and testosterone observed *in vivo* by Jensen et al. (2001) agrees well with FBA predictions presented in this study (Fig. 5). Experimental evidence for changes in levels of progestogens and corticoids over a fathead minnow spawning cycle are not presently available. *In silico* predictions during the PreVtg-Vtg stages show progestogen productions to be highest, followed by corticoids, androgens and estrogens (Fig. 5). While FBA predictions cannot be directly verified against concomitant oogenesis stages *in vivo*, previous work by the author has shown *ex vivo* ovarian hormone productions from female fathead minnows to show highest production for progestogens (0.26 ± 0.2 fmol/mg/hr), followed by androgens (0.17 ± 0.1 fmol/mg/hr), estrogens (0.14 ± 0.1 fmol/mg/hr) and a corticoid (0.06 fmol/mg/hr) (Hala and Huggett, 2014). Hala and Huggett (2014) monitored productions of four progestogens (progesterone, pregnenolone, 17α-hydroxyprogesterone and 17α-hydroxypregnenolone), two androgens (11-ketotestosterone and testosterone), two estrogens (17β-estradiol and estrone) and one corticoid (11-deoxycortisol).

Finally, the CA analysis of Log_{10} transformed flux values showed early oogenesis stages (including PreVtg-Vtg and Vtg-Mature) to exhibit high overlap, indicating homologous variance (Fig. 3(b)). This was explainable by homologous variances for reactions producing progestogens (47 and 49), androgens (46, 48, 83, 84, 97 and 98), estrogen (99) and corticoid (50) (**Supplemental 2: Oogenesis-CA Flux Contribution**). Mid to late oogenesis stages constituting Mature-Ovulated and Ovulated-Atretic stages were dispersed across the first CA axis (which accounted for 99.8% of variance) (Fig. 3(b)). The juxtaposition of Mature-Ovulated vs. Ovulated-Atretic stages across the negative and positive orthant of the first CA axis respectively alludes to variable flux constraints. Specifically, fluxes associated with reactions 2 and 26 (producing pregnenolone and progesterone respectively) were better able to explain variance for the Mature-Ovulated stage (**Supplemental 2: Oogenesis-CA Flux Contribution**). The high involvement of progestogen synthesis reactions during Vtg-Mature and Mature-Ovulated stages (i.e. fluxes for reactions 47, 49, 2 and 26) is consistent with their prominent involvement during oocyte maturation and ovulation (Nagahama and Yamashita, 2008). Whereas, fluxes for reactions 73 and 74 (producing 11β-hydroxytestosterone and 11-ketotestosterone respectively) had more impact on the final (Ovulated-Atretic) stage of oogenesis (Fig. 3(b)). Therefore, despite the fact that *in silico* predicted steroid enzyme gene activations for the final oogenesis stage (Ovulated-Atretic) appear to revert back to an initial configuration (PreVtg-Vtg) (Fig. 4), the flux constraints associated with the two stages are markedly different. However, this difference in internal flux states does not appear to impact steroid hormone productions at the Ovulated-Atretic stage, as levels for progestogens, corticoids, androgens and estrogens appear to revert back to levels typical of oogenesis initiation (PreVtg-Vtg stage) (Fig. 5). As previously discussed in Section 3.3, it is likely that increased availabilities of TFs at the final Ovulated-Atretic stage help prepare for a re-initialization to the PreVtg-Vtg stage and elevated productions of steroid hormones, which appears to be an *in vivo* hallmark of early oogenesis stages in reproducing female fathead minnows (Jensen et al., 2001).

3.5. Limits and relevance of model predictions

Keeping in mind the often paraphrased adage on the accuracy and usefulness of models (Box, 1976), it is important to consider the limits and relevance of the models (and associated predictions) presented in this study. Boolean rules used to parameterize the TRN model are a 'best representation' of current knowledge on the regulation of vertebrate steroidogenic genes, and are not species-specific. Therefore, tailored models specific to the organism under study are needed, and will require comprehensive characterizations of genome-wide TF bindings (using chromatin immunoprecipitation and sequencing). Furthermore, use of the Boolean OR operator created a flexible conditional logic in which the sole presence of either conditional TF could lead to gene activation. Therefore, considered solely at the transcriptional level, one might expect almost any gene to be consistently active given fulfilment of either conformist/contrarian TF presence/absence. Regardless of this generalization, however, extreme pathway analysis enabled assessment of the extent or 'magnitude' of gene activations subject to condition-specific presence/absence of TFs. For example, if a gene were to be fully active, its activation-state would be represented in all extreme pathway vectors calculated for any given condition. This propensity was utilized to calculate steroido-

genic gene activation magnitudes during female fathead minnow oogenesis, enabling the generation of scalars to re-constrain reaction inequality bounds in FBA steroidogenesis models (Fig. 1). The inter-relation of TRN model outputs (i.e. magnitude gene expression changes) with FBA model inputs (re-constrained inequality bounds), allowed construction of multilevel models of oogenesis. However, the premise of such inter-relation implies close correspondence between transcriptomic changes and metabolic responses. The validity of this assumption needs to be considered in more detail.

Overall, correspondence between gene expression and associated peptide productions show weak correlation coefficients from ~0.3 to >0.5 (Arvas et al., 2011; de Sousa Abreu et al., 2009; Ghazalpour et al., 2011). Such weak correlations are likely due to variations in post-translational modifications and/or peptide half-lives (along with analytical error or noise) (Greenbaum et al., 2003). Interestingly, stronger correlations are observed if mRNA and corresponding peptides are from the same gene. Therefore, genes constituting functionally inducible systems (or pathways) can be expected to have strongly correlated expressions and associated peptide productions (Wang et al., 2010). While not comprehensively investigated, steroidogenesis in synchronously spawning piscine species demonstrate such a close correspondence between gonadal steroidogenic enzyme gene expressions and associated steroid hormone productions during reproduction (Kumar et al., 2000). Studies with asynchronously spawning fathead minnows also show such correspondence with elevated gene expressions during spawning correlating with increased steroid hormone titers (Jensen et al., 2001; Villeneuve et al., 2010). This close association between gene expression and hormone productions is often used in toxicological studies in which gene expression changes are used as diagnostic biomarkers of reproductive toxicity in fathead minnows (Ankley et al., 2009). Therefore, functional coordination between gene expression and hormone productions during fathead minnow reproduction lends credence to the integrated *in silico* approach taken in this manuscript. The results presented show both TRN and FBA models to represent and reflect key functional aspects of fathead minnow oogenesis. Looking ahead, model performance may be improved by incorporating ternary logic within Boolean rules to account for more variable expressions for TF genes (i.e. low, mid, high expressions), and incorporating hormone feedback control of genes in a regulatory or dynamic FBA framework (Covert et al., 2001). Such changes will allow the discrete-dynamic projection of steroidogenic function and allow analysis of stable vs. un-stable functional states over ontogeny and/or spawning cycles.

4. Summary and conclusions

This manuscript used an integrated *in silico* computational framework to study the transcriptional regulatory control of steroidogenesis during oogenesis in female fathead minnows. Two stoichiometric models were constructed. The first was a transcriptional regulatory network (TRN) model of steroidogenic enzyme gene regulation in ovary tissue. The second was a mass-balanced stoichiometric model of steroidogenesis. The TRN model was parameterized with the presence/absence of transcription factors (TFs) responsible for regulating steroidogenic enzyme genes during distinctive oogenesis stages (Villeneuve et al., 2010). Once parameterized, extreme pathway (ExPa) analysis was used to predict the underlying magnitude of gene expression changes for steroidogenic enzyme genes during oogenesis. Analyses showed overall elevated gene expressions during early oogenesis, with subsequently decreasing expressions until maturation-ovulation stages, and a return of expressions back to elevated levels post-ovulation. The magnitude change in gene expressions was used to re-constrain inequality bounds for steroidogenic enzyme catalyzed reactions in the steroidogenesis model. Flux balance analysis (FBA) was used to predict steroid hormone productions during oogenesis. Productions of all hormones tracked gene expression changes during oogenesis. Analyses showed elevated synthesis of progestogens, followed by corticoids, androgens and estrogens during early oogenesis. These levels decreased during mid-oogenesis (Mature-Ovulated stages) and reverted back to elevated productions post-ovulation (Ovulated-Atretic stage). *In silico* simulations from early to mid-oogenesis stages agree well with *in vivo* experimentation using spawning female fathead minnows. While, Villeneuve et al. (2010) show elevated steroidogenic enzyme gene expressions during early oogenesis (with lowered expressions thereafter), Jensen et al. (2001) show concomitant increases in steroid hormone levels (for testosterone and 17β-estradiol) during early oogenesis, with decreasing levels as oogenesis progressed. Therefore, our integrated modelling approach is capable of representing some of the important functional aspects of reproduction in fish, and provides a computational framework for exploring effects of adverse environmental and anthropogenic stressors on piscine fecundity.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jtbi.2018.07.020.

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