Estrogenic chemicals in the aquatic environment have been shown to cause a variety of reproductive anomalies in fish including full sex reversal, intersex, and altered population sex ratios. Two estrogens found in the aquatic environment, 17α-ethinylestradiol (EE2) and 17β-estradiol (E2), have been measured in wastewater treatment effluents and have been shown to cause adverse effects in fish. To further our understanding of how estrogen exposure affects reproductive endpoints in the male fathead minnow (FHM, Pimephales promelas), a physiologically based computational model was developed of the hypothalamic-pituitary-gonadal (HPG) axis. Apical reproductive endpoints in the model include plasma steroid hormone and vitellogenin ratios. Two estrogens found in the aquatic environment, 17α-ethinylestradiol (EE2) and 17β-estradiol (E2), have been measured in wastewater treatment effluents and have been shown to cause adverse effects in fish. To further our understanding of how estrogen exposure affects reproductive endpoints in the male fathead minnow (FHM, Pimephales promelas), a physiologically based computational model was developed of the hypothalamic-pituitary-gonadal (HPG) axis. Apical reproductive endpoints in the model include plasma steroid hormone and vitellogenin concentrations. Using Markov chain Monte Carlo simulation, the model was calibrated with data from unexposed FHM, and FHM exposed to EE2 and E2. Independent experimental data sets were used to evaluate model predictions. We found good agreement between our model predictions and a variety of measured reproductive endpoints, although the model underpredicts unexposed FHM reproductive endpoint variances, and overpredicts variances in estrogen-exposed FHM. We conclude that this model provides a robust representation of the HPG axis in male FHM.

Key Words: EE2; E2; fish; steroid hormones; environmental estrogen; Markov chain Monte Carlo simulation; predictive toxicology; system model.

The vertebrate endocrine system regulates processes vital for reproduction, growth, and metabolism through an array of biochemical signals and their receptors (Norris, 1997). Endocrine tissues and glands (e.g., hypothalamus, pituitary, thyroid, and adrenal glands) produce hormones that circulate in the bloodstream of an organism and regulate processes in tissues and organs throughout the body. Within the endocrine system, the hypothalamic-pituitary-gonadal (HPG) axis is viewed as the primary subsystem regulating processes important for reproduction, and is the focus of the computational model described in this paper. This dynamic, complex regulatory system is well understood in several mammalian species, but much less is known about endocrine systems in lower vertebrates such as fish.

Endocrine signaling can be altered by a variety of exogenous chemicals collectively termed endocrine disrupting chemicals (EDCs), which mimic or block the activity of endogenous hormones through binding to their receptors, or they alter the production or availability of natural hormones. Fish and other aquatic organisms can be exposed to chemicals throughout their lifetime, and survival of these species is particularly vulnerable to adverse effects from these agents on reproduction and development (Kidd et al., 2007). In addition, fish exposed to wastewater treatment plant effluents initially described in the United Kingdom, and subsequently elsewhere have been found with gross reproductive abnormalities (e.g., ovo-testis), altered steroid hormone levels, altered male:female population sex ratios, and increased plasma concentrations of vitellogenin (Vtg), a precursor to a major egg yolk protein (Filby et al., 2007a; Jobling et al., 2002; Liney et al., 2006; Orlando et al., 2004; Sumpter and Johnson, 2005; Vajda et al., 2008; Woodling et al., 2006). These adverse effects have been attributed to estrogenic EDCs such as 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) (Folmar et al., 2002; Kang et al., 2002; Öm et al., 2006; Parks et al., 1999; Seki et al., 2002; Van den Belt et al., 2003; Versonn and Janssen, 2004).

EE2 is a synthetic estrogen used in birth control pills, and it has a higher binding affinity for the estrogen receptor (ER) than E2 (Matthews et al., 2000), the endogenous ligand. Both EE2 and E2 have been found in the aquatic environment at levels...
that range from 0.5 to 15 ng EE\textsubscript{2}/l (Desbrow et al., 1998; Ericson et al., 2002; Ribeiro et al., 2008; Ying et al., 2008), and < 1 to 48 ng E\textsubscript{2}/l (Desbrow et al., 1998; Labadie and Budzinski, 2005; Ribeiro et al., 2008; Ying et al., 2008). In a recent study, Kidd et al. (2007) found that additions of E\textsubscript{2} to an experimental lake yielding water concentrations of approximately 5 ng E\textsubscript{2}/l resulted in a collapse of the native FHM population about 1.5 years (in Fall 2002) after the start of E\textsubscript{2} additions to the lake. Moreover, in laboratory studies, environmentally relevant concentrations of either EE\textsubscript{2} or E\textsubscript{2} have been shown to affect function of the HPG axis in fish (Brion et al., 2004; Halm et al., 2002; Lange et al., 2001; Panter et al., 2000; Parrott and Blunt, 2005; Pawlowski et al., 2004). Most laboratory studies focus on exposure to individual estrogens, whereas in the aquatic environment mixtures prevail. Some studies with FHMs have been shown to involve mixtures of estrogens, including E\textsubscript{2} and EE\textsubscript{2}, which can affect key HPG-mediated processes in fish, including reproduction. This understanding would be greatly facilitated by a computational model describing the interaction of estrogens with the fish HPG axis.

To improve our understanding of how estrogenic EDCs affect reproductive endpoints, we developed a physiologically based computational model of the HPG axis for male FHMs exposed to EE\textsubscript{2} and E\textsubscript{2}. This model is based upon published graphical systems model described by Villeneuve et al. (2007), and simulates key reproductive endpoints such as plasma concentrations of testosterone (T), 11-ketotestosterone (KT), E\textsubscript{2}, and Vtg. Our model is different from prior models of the HPG axis in fish (Kim et al., 2006; Murphy et al., 2005; Schultz et al., 2001), rats (Barton and Andersen, 1998; Inoue et al., 1970; Schlosser et al., 2006; Teegarden and Barton, 2004), and humans (Enciso and Sontag, 2004; Plouffe and Luxenberg, 1992) because it is able to simulate responses for both EE\textsubscript{2} and E\textsubscript{2} exposure. Furthermore, we utilize a probabilistic, Markov chain Monte Carlo (MCMC) method of model calibration that accounts for natural biological variability and provides model predictions with confidence intervals (CIs).

### MATERIALS AND METHODS

#### Computational Model Formulation

Our physiologically based model simulates the absorption, distribution, and elimination of EE\textsubscript{2} and endogenous hormones of the HPG axis in male FHM. Though a more detailed conceptual model has been described by Villeneuve et al. (2007), the data needed to parameterize a corresponding computational model are currently unavailable. We simplified many of the biochemical processes reported in Villeneuve et al., and incorporated these processes into a model that contains six compartments (i.e., gill, brain, gonad, liver, venous blood, and "other") involved in HPG axis signaling and EE\textsubscript{2} disposition (Fig. 1). Following principles of pharmacokinetic modeling, for each compartment and chemical of interest, a mass balance was formulated for the free (or unbound) chemicals of interest (see Supplementary Data Eq. 1) to create a set of coupled ordinary differential equations that comprise the computational model. Total chemical concentrations were computed as the sum of free and specifically bound chemical concentrations (Supplementary Data Eq. 3). A comprehensive description of the model formulation with equations can be found in the online supplement.

#### Gill

The gill compartment is where exogenous E\textsubscript{2} uptake occurs from the aquatic environment. As water containing EE\textsubscript{2} flows across the gills, EE\textsubscript{2} is taken up into arterial blood that distributes throughout the body. In zebrafish, steroid binding proteins (SBPs) in gill have been found to facilitate EE\textsubscript{2} uptake from water (Miguel-Queralt and Hammond, 2008). In our model, EE\textsubscript{2} uptake is formulated using an equilibrium partition coefficient between blood and water, which simplifies the EE\textsubscript{2} uptake process and enables the entrance of EE\textsubscript{2} into the bloodstream. Similarly, we use partition coefficients to represent the fraction of EE\textsubscript{2} that leaves the blood to enter different tissues and organs (Supplementary Data Eq. 5).

#### Brain

The brain regulates the HPG axis by producing hormones that circulate to target tissues (e.g., gonad). In the brain compartment, gonadotropin releasing hormone (GnRH) regulates the release of luteinizing hormone (LH, or gonadotropin II) (Yaron et al., 2003), but the dynamics of GnRH were not included in this model because GnRH data were unavailable. Instead, we represented the periodic nature of GnRH dynamics and its effect on LH by producing LH in brain according to a periodic function, which is similar to assumptions made by Murphy et al. (2005). Basal LH production is formulated as a periodic step function of 12 h on and 12 h off (Supplementary Data Fig. S2). In addition, LH production can be stimulated by E\textsubscript{2} (Yaron et al., 2003), which is formulated as a positive feedback in our model, although Murphy et al. (2005) formulated the effect of E\textsubscript{2} on LH production as a negative feedback for Atlantic croaker. Our model simulates the production of E\textsubscript{2} from T that circulates to the brain in blood. Also, the model is formulated such that E\textsubscript{2} in the brain is able to bind to ERs, which serves as a "sink" in the brain’s E\textsubscript{2} mass balance. Hormones produced in the brain are secreted into the blood and circulate throughout the body.

#### Gonad

A target tissue of LH is the gonad where LH binds to LH receptors to regulate steroidogenesis (Senthilkumar et al., 2004). In our model, LH bound to LH receptors stimulates the production of steroidogenic acute regulatory protein (Star), a carrier protein that transports cholesterol from cytosol into mitochondria. The quantity of cholesterol available for steroidogenesis is formulated to be proportional to the quantity of Star produced (Nunez and Evans, 2007). Cholesterol is the initial substrate in a cascade of reactions that produce T (Halm et al., 2002; Nagahama et al., 1994, 1995; Senthilkumar et al., 2004). A more detailed computational model of steroidogenesis exists for FHM (Breen et al., 2007), however, we could not implement it here because concentrations of intermediate steroid hormones are unavailable. Thus, we simplified the complex process of T production from cholesterol by lumping the multiple reactions into one “effective” reaction (Murphy et al., 2005). This “effective” reaction was formulated as a Hill equation (Supplementary Data Eq. 12). Production of E\textsubscript{2} and KT from T is formulated as a Michaelis-Menten equation (Supplementary Data Eq. 14 and Eq. 15). Because a decrease in plasma T concentration was observed after EE\textsubscript{2} exposure, we assumed an autocrine/paracrine negative feedback process in the gonad (Callard, 1992) where E\textsubscript{2} or EE\textsubscript{2} binds to ER, and the E\textsubscript{2}-ER or EE\textsubscript{2}-ER complex inhibits T production (Supplementary Data Eq. 13). Hormones produced in the gonad are secreted into blood and circulate throughout the body.

#### Liver

When estrogens such as E\textsubscript{2} and EE\textsubscript{2} enter the liver, they bind to ERs forming E\textsubscript{2}-ER and EE\textsubscript{2}-ER complexes. These complexes then bind to estrogen response elements (EREs) of target genes (e.g., ER and Vtg) to regulate gene expression. The dominant ER subtype in FHM liver is ER\textsubscript{x}, which is the only ER subtype known to be induced by E\textsubscript{2} or EE\textsubscript{2} (Filby and Tyler, 2005; Filby et al., 2007b). In rainbow trout hepatocyte cultures, Flouriot et al. (1996, 1997) found that when exogenous E\textsubscript{2} concentration was zero, ER mRNA was...
expressed at low levels while Vtg mRNA was not expressed. As a function of increasing exogenous E2 concentration, both ER and Vtg gene expression increased in a dose-dependent manner (Flouriot et al., 1996). Assuming that ER gene expression correlates with ER protein, in our model bound ER is formulated to increase the production of both ER itself and Vtg. For example, we used measured changes in ER gene expression to provide limits to changes in ER protein induction. Moreover, we are modeling all ERs based on data from ERα. The ER kinetics in liver include background production, induction...
by bound ER, association and dissociation with E₂ and EE₂, and elimination (see Supplementary Data Eq. 16).

In male FHMs, plasma Vtg concentrations are normally very low, and are usually undetectable at the μg/ml level. Exposure to estrogens such as EE₂ has been shown to cause abnormal hepatic production of Vtg, and raise plasma Vtg concentrations to unusually high levels (Filby and Tyler, 2005; Latter et al., 2002; Schmid et al., 2002). In our model, we associated the production rate of Vtg protein in liver with the concentration of E₂-ER and EE₂-ER complexes (Supplementary Data Eq. 17). Vtg produced in liver is secreted into blood and circulates throughout the body.

We did not include steroid hormone or EE₂ metabolism reactions, although they have been identified in other fish species (Ohkamoto et al., 2003; Snowberger and Stegemann, 1987). Liver metabolism serves to eliminate a parent compound from the body, and multiple reactions would need to be added to the model with sufficient data to provide model parameter values. Adding these reactions would only increase the uncertainty and complexity of the model without a significant improvement in model predictions. Thus, we formulated steroid hormone, EE₂, and Vtg elimination as first-order elimination processes in the “other” compartment (see description of the “other” compartment for details).

**Venous blood.** In the blood of most vertebrates E₂ and T are predominantly bound to SBPs, which modulate steroid hormone bioavailability and distribution to target tissues (Toeguarden and Barton, 2004; Tollefsen et al., 2004). In our model, free and SBP-bound T and E₂ concentrations are simulated in venous and arterial blood compartments (Supplementary Data equations for venous blood). KT also binds to SBPs, but with a much lower binding affinity than E₂ and T based on studies in other fish species (Laidley and Thomas, 1994; Miguel-Queralt and Hammond, 2008; Pasmanik and Callard, 1986). For E₂, Tollefsen et al. (2004) reported that EE₂ binding to Arctic char (*Salvelinus alpinus*) SBP was approximately 400-fold less than that of E₂. However, Gale et al. (2004) reported that the binding affinity of EE₂ for channel catfish (*Ictalurus punctatus*) SBP was threefold higher than that of E₂. More recently, Miguel-Queralt and Hammond (2008) reported that the binding affinity of EE₂ to recombinant SBP from zebrafish is fivefold higher than that of E₂. These studies (Gale et al., 2004; Miguel-Queralt and Hammond, 2008; Tollefsen et al., 2004) suggest large variability in the binding affinity of EE₂ to SBPs, which may be a result of different fish species or different test methods. Inclusion of EE₂ binding to SBPs in our model would allow EE₂ to compete and displace E₂ and T from SBPs in blood, and as a result free E₂ and free T concentrations in blood would increase. However, the increase in free E₂ and T would not be significant because the total SBP concentration was defined as 20μM, a relatively low value. Thus, KT and EE₂ binding to SBPs was not included in this model.

**Other.** In our model all tissues except for gill, brain, gonad and liver were grouped into a compartment called “other.” A unique function of the “other” compartment is the elimination of T, KT, E₂, and Vtg according to first-order kinetics (Supplementary Data Eq. 19). This is a simplifying assumption since Vermeirssen and Scott (1996) showed that rainbow trout steroid hormones, mainly in conjugated form, are excreted in urine and bile. However, without data to parameterize and calibrate kinetic equations of steroid hormone biotransformation and conjugation in liver and subsequent elimination by the kidney, representing these complex reactions would introduce more uncertainty into the model. That is, the kinetic rate constants for these reactions could take on any value as long as the steroid hormone production parameters compensated for the increase or decrease in elimination. Thus, we chose to represent the elimination of steroid hormones, EE₂, and Vtg with first-order kinetic processes in the “other” compartment where the kidney resides.

**Experimental Data**

Reproductive endpoint data in unexposed FHMs were obtained from over 10 experiments conducted at the USEPA Duluth laboratory from 1999 to 2006, which were summarized in Watanabe et al. (2007). In total, there were 143 unexposed (control) male FHMs associated with these experiments. The data for 70 unexposed fish were used to calibrate the model and the remainder was used to evaluate the model. For each individual fish, measured model input parameters included body weight (BW), gonad weight (GW), and liver weight (LW). Model predictions of T, KT, E₂, and Vtg plasma concentrations were compared with measurements of T, KT, E₂, and Vtg through a likelihood function (Gelman et al., 1995). Summary statistics of the unexposed FHM data used to calibrate the model are reported in Table 1.

<table>
<thead>
<tr>
<th>Statistics</th>
<th>BW (g)</th>
<th>GW (mg)</th>
<th>LW (mg)</th>
<th>T (ng/ml)</th>
<th>KT (ng/ml)</th>
<th>E₂ (ng/ml)</th>
<th>Vtg (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3.80</td>
<td>1.15</td>
<td>38</td>
<td>1.39</td>
<td>9.38</td>
<td>33.08</td>
<td>0.50</td>
</tr>
<tr>
<td>SD</td>
<td>0.85</td>
<td>0.38</td>
<td>11</td>
<td>0.21</td>
<td>5.00</td>
<td>20.66</td>
<td>0.37</td>
</tr>
<tr>
<td>Median</td>
<td>3.78</td>
<td>4.06</td>
<td>38</td>
<td>1.45</td>
<td>8.84</td>
<td>29.65</td>
<td>0.40</td>
</tr>
<tr>
<td>Maximum</td>
<td>5.48</td>
<td>2.19</td>
<td>54</td>
<td>1.63</td>
<td>24.12</td>
<td>70.05</td>
<td>1.60</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.91</td>
<td>0.35</td>
<td>20</td>
<td>1.05</td>
<td>2.82</td>
<td>2.49</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Note. GSI = gonadosomatic index, equal to (GW/BW) × 100; HSI = hepatosomatic index, equal to (LW/BW) × 100.

Data for EE₂-exposed male FHMs were obtained from two studies: (1) a 48-h static exposure to concentrations of 10 and 50 ng EE₂/l (Garcia-Reyero et al., Forthcoming); and (2) a continuous, flow-through exposure to concentrations of 10 and 100 ng EE₂/l (Ekmann et al., 2008). Actual measured EE₂ water concentrations from the two studies were used as input into the model. In Garcia-Reyero et al. (Forthcoming), male FHMs were exposed to 10 and 50 ng EE₂/l for 48 h, and plasma T and Vtg concentrations were measured in four fish per exposure concentration (Table 2). As part of the study by Garcia-Reyero et al. (Forthcoming) in male FHMs, plasma EE₂ concentrations in carcass and liver also were measured in pooled tissues from 30 male FHMs exposed to 50 ng EE₂/l for 48 h (unpublished data). After exposure, the average total EE₂ liver concentration was 57.8 ± 29.5 ng/g dry tissue, and the average total EE₂ carcass concentration was 13.9 ± 3.7 ng/g dry tissue. In Ekmann et al. (2008), plasma Vtg concentrations in male FHMs were measured in eight males per sampling period after days 1, 4, and 8 of exposure to EE₂ and 8 days after EE₂ exposure (test day 16). Data from Garcia-Reyero et al. (Forthcoming) were used for model calibration, and data from Ekmann et al. (2008) were used for model evaluation.

Data from E₂-exposed male FHMs were obtained from two studies: (1) Korte et al. (2000); and (2) Parks et al. (1999). Korte et al. (2000) injected 97 male FHMs with 250 μg E₂/l for 48 h and measured plasma E₂ concentrations.

**TABLE 1**

Summary Statistics of Baseline Data from Male FHM (*n* = 70) Used in Model Calibration

<table>
<thead>
<tr>
<th>Statistics</th>
<th>BW (g)</th>
<th>GW (mg)</th>
<th>LW (mg)</th>
<th>T (ng/ml)</th>
<th>KT (ng/ml)</th>
<th>E₂ (ng/ml)</th>
<th>Vtg (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3.80</td>
<td>1.15</td>
<td>38</td>
<td>1.39</td>
<td>9.38</td>
<td>33.08</td>
<td>0.50</td>
</tr>
<tr>
<td>SD</td>
<td>0.85</td>
<td>0.38</td>
<td>11</td>
<td>0.21</td>
<td>5.00</td>
<td>20.66</td>
<td>0.37</td>
</tr>
<tr>
<td>Median</td>
<td>3.78</td>
<td>4.06</td>
<td>38</td>
<td>1.45</td>
<td>8.84</td>
<td>29.65</td>
<td>0.40</td>
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<tr>
<td>Maximum</td>
<td>5.48</td>
<td>2.19</td>
<td>54</td>
<td>1.63</td>
<td>24.12</td>
<td>70.05</td>
<td>1.60</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.91</td>
<td>0.35</td>
<td>20</td>
<td>1.05</td>
<td>2.82</td>
<td>2.49</td>
<td>0.10</td>
</tr>
</tbody>
</table>

**TABLE 2**

Plasma T and Vtg Concentrations Measured in Male FHM Exposed to Nominal Water Concentrations of 10 or 50 ng EE₂/l for 48 h (Garcia-Reyero et al., Forthcoming)

<table>
<thead>
<tr>
<th>Fish ID</th>
<th>BW (g)</th>
<th>T (ng/ml)</th>
<th>Vtg (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal water concentration = 10 ng EE₂/l, average measured concentration = 5.3 ng EE₂/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3.9</td>
<td>2.2</td>
<td>29</td>
</tr>
<tr>
<td>SD</td>
<td>0.78</td>
<td>0.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

| Nominal water concentration = 50 ng EE₂/l, average measured concentration = 41 ng EE₂/l |
| Mean    | 3.4    | 2.5       | 29          |
| SD      | 0.85   | 0.38      | 16          |

| Nominal water concentration = 10 ng EE₂/l, average measured concentration = 50 ng EE₂/l |
| Mean    | 3.9    | 2.2       | 29          |
| SD      | 0.78   | 0.2       | 3.1         |

FHMs with 0.5 or 5 mg E2/kg, and measured plasma E2 and Vtg concentrations at 10 different times over 18 days (n = 5 fish per sample). Parks et al. (1999) injected three male FHMs with 25 mg E2/kg on days 0, 2, and 4, and measured plasma E2 concentrations on day 7. The Korte et al. (2000) data were used to calibrate the model and Parks et al. (1999) data were used to evaluate our model predictions.

Model Calibration

The model requires information about experimental conditions and 82 model parameters such as tissue compartment volumes, blood flow rates, equilibrium partition coefficients, and rate constants for chemical reactions. Sixty-two model parameters were fixed at values listed in Table S1 (Supplementary Data) because they were either (1) measured directly in FHM; (2) scaled allometrically to FHM from data in other species; (3) insensitive parameters with respect to the available measured data; or (4) they could not be uniquely identified given the other model parameters being calibrated and the available data. The remaining 20 model parameters (Table 3) were calibrated with data collected in FHMs. To account for biological uncertainty and variability, we used a probabilistic, Bayesian approach called MCMC simulation to calibrate 20 model parameters (see Table 3) following methods described in previous studies (Bois et al., 1996b; Gelman et al., 1995; Lin et al., 2004; Watanabe et al., 2005). MCMC simulation is a computational method based on Bayes’ rule that provides a joint posterior distribution of model parameters that is proportional to the product of the parameter prior distributions and the data likelihood. This method has advantages over other parameter optimization methods in that it: (1) provides a method of incorporating existing knowledge about a range of possible parameter values through specification of a prior distribution; (2) accounts for covariance between the 20 calibrated model parameters; and (3) allows for determination of model prediction CIs. To perform MCMC simulation, one must specify prior parameter distributions, and experimental conditions and data for computing the likelihood function as described below.

We specified model parameter prior distributions based on data available in the literature. When little or no data were available, we assigned a vague prior distribution such as a uniform or log-uniform distribution with a large range to allow the experimental data to refine the posterior parameter distribution. For example, we could not find a published value for the Hill coefficient for Vtg production in liver, but we assumed that it would be approximately two based on the dimerization of ER complexes needed to stimulate gene expression. Thus, we assigned a log-uniform distribution with a lower bound of zero and an upper bound of 10 (Table 3). Similarly, an EE2 partition coefficient for blood to water was reported for rainbow trout (Kim, 2004), but not FHM, so we assigned a log-normal distribution with a geometric mean of 600 and a geometric standard deviation of three, which corresponds to a coefficient of variation equal to 1.5.

Two of the 20 calibrated parameters were the variances of measured plasma T and E2 concentrations. Measurements were made for these two endpoints in unexposed and E2- or EE2-exposed FHMs, but relatively few measurements were made in the exposed FHMs. Thus, the plasma T and E2 concentration variances were treated as unknowns and calibrated with MCMC simulations. Following methods used previously (Bois et al., 1996b; Lin et al., 2004) inverse gamma prior distributions were assigned to the variances based on a natural logarithm transformation of the measured plasma E2 and T concentrations.

Simulation of EE2 or E2 exposure experiments with our model began at model simulation time equal to 3600 h in order to allow the model to reach homeostasis/steady-state before starting the exposure. Starting an experimental exposure at 3600 h was necessary because at the start of a simulation (i.e., 0 h), most model output variables (e.g., plasma T, KT, and E2 concentrations) were set to equal zero because they have not been measured at birth. For each simulated experiment, the exposure duration was defined by the experimental conditions, and model predictions were made at times corresponding to each measurement. Thus, a 48-h exposure to EE2 was started at 3600 h and ended at 3648 h; plasma Vtg predictions were output at 3648 h to correspond to measured plasma Vtg concentrations at the end of the 48-h exposure period. MCMC simulations were performed with MCSim (Bois and Massele, 1997), a software package freely available online (http://directory.fsf.org/math/mcsim.html), using the Metropolis-Hastings algorithm. Four independent Markov chains were run that started with parameter values ran selected from their assigned prior distributions. Simulations were performed until the four chains converged for all 20 parameters. Convergence was assessed by the potential scale reduction method (Gelman et al., 1995) that compares the variance between and within Markov chains for the last n values from each chain (n = 1000 in this study). As recommended by Gelman et al. (1995), we used potential scale reduction values between 1.0 and 1.2 as our criteria for convergence.

RESULTS

Model Calibration

We evaluated the predictive ability of our model by simulating plasma Vtg concentrations from two studies that used different experimental (including exposure) conditions compared with the studies used to calibrate the model. Four thousand sets of 20 calibrated model parameters were used to simulate two different experiments from Ekman et al. (2008) (see Experimental Data section). Exposure to EE2 was started at simulation time equal to 3600 h for reasons described in the Model Calibration section. Simulated exposures to 10 and 100 ng E2/kg lasted for 8 days, and plasma Vtg concentrations were output at days 1, 4, 8, and 16 (i.e., 8 days postexposure). We also simulated the plasma concentrations of Vtg in male FHMs 7 days after three doses of E2 injection, and compared the predictions to measurements from an independent study by Parks et al. (1999). Parks et al. reported that the plasma concentration of Vtg on the seventh day after three injections of E2 on days 0, 2, and 4 was 21.3 ± 1.3 mg/ml. The model predictions were compared with the measured data visually.

Four separate Markov chains of 6500 iterations were run in order to reach convergence for all 20 parameters treated as unknown random variables. The speed of each chain was approximately 90 h per 1000 iterations on a 2.8-GHz Linux workstation. Figure 2 plots the progression of parameter values for two of the 20 calibrated parameters: (a) blood to water partition coefficient for EE2 uptake; and (b) Hill coefficient for Vtg production in liver. For these two parameters, convergence or mixing of the four Markov chains is achieved much earlier than the 6,500 iterations needed for all 20 parameters to converge. Summary statistics for posterior parameter values (the last 1000 parameter sets from each Markov chain for a total of 4000) are reported in Table 3. Two calibrated model parameters $K_d_{E2ER,brn}$, $\rho_{Chol,gon}$ had posterior 95% CIs slightly larger than their prior distribution 95% CIs; three calibrated parameters ($k_{E2ER,brm}$, $\rho_{LH,brm}$, $\rho_{STAR,gon}$) had similar prior and posterior 95% CIs; and 14 calibrated model parameters had posterior 95% CIs smaller than their prior distribution 95% CIs. One calibrated parameter, the variance of the natural logarithm of plasma E2 concentration (Var_Ln_CE2tot_pla_ngml), had a posterior 95% CI that did not overlap with the 95% CI of its prior distribution because its assigned prior distribution was based on plasma E2 concentrations from unexposed control fish only. Values of the posterior distribution of Var_Ln_CE2tot_pla_ngml are consistent with the variance in the data used to calibrate with model, which include both unexposed control FHM and FHM injected with E2 in Korte et al. (2000). That is, the variance of the
<table>
<thead>
<tr>
<th>Parameter description</th>
<th>Symbols</th>
<th>Prior distribution (P1, P2)*</th>
<th>Reference</th>
<th>Mean</th>
<th>Median</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnitude of LH production (nmol/h)</td>
<td>MagLH</td>
<td>Log-uniform (5.0e-06, 5.0e-04)</td>
<td>Schulz et al. (1993)</td>
<td>2.6e-5</td>
<td>2.6e-5</td>
<td>(9.2e-6, 4.0e-5)</td>
</tr>
<tr>
<td>Dissociation constant of E2 binding to ER in brain (nM)</td>
<td>K_d,E2ER, bm</td>
<td>Log-normal (2.5, 3)</td>
<td>Murphy et al. (2005)</td>
<td>7.3</td>
<td>2.8</td>
<td>(0.24, 57)</td>
</tr>
<tr>
<td>Association rate of E2 binding to ER in brain (1/nM/h)</td>
<td>k_1,E2ER, bm</td>
<td>Log-normal (0.743, 3)</td>
<td>Murphy et al. (2005)</td>
<td>1.7</td>
<td>0.84</td>
<td>(0.094, 10)</td>
</tr>
<tr>
<td>Association rate of LH binding to LH receptor in gonad (1/nM/h)</td>
<td>k_1,LH,LH, gon</td>
<td>Log-normal (0.1, 3)</td>
<td>Assumed</td>
<td>0.20</td>
<td>0.12</td>
<td>(0.015, 0.87)</td>
</tr>
<tr>
<td>Induction rate of LH production by E2 (1/h)</td>
<td>pLH,brn</td>
<td>Log-uniform (0.0005, 0.5)</td>
<td>Assumed</td>
<td>0.19</td>
<td>0.18</td>
<td>(7.0e-4, 0.47)</td>
</tr>
<tr>
<td>Proportionality constant relating bound LH receptor to STAR</td>
<td>pSTAR,gon</td>
<td>Log-uniform (1, 100)</td>
<td>Assumed</td>
<td>30</td>
<td>22</td>
<td>(1.3, 91)</td>
</tr>
<tr>
<td>Proportionality constant relating cholesterol to STAR</td>
<td>pChol,gon</td>
<td>Log-uniform (10, 10,000)</td>
<td>Artemenko et al. (2001)</td>
<td>2.6e+3</td>
<td>1.5e+3</td>
<td>(57, 9.0e +3)</td>
</tr>
<tr>
<td>Elimination rate constant for E2 in the ‘‘other’’ compartment (1/h)</td>
<td>KelimE2,oth</td>
<td>Log-normal (0.1, 3)</td>
<td>Teeguarden and Barton (2004)</td>
<td>0.28</td>
<td>0.28</td>
<td>(0.21, 0.36)</td>
</tr>
<tr>
<td>ER background production rate (nM/h)</td>
<td>PbgER,liv</td>
<td>Log-uniform (5.0e-5, 5.0e-1)</td>
<td>Assumed</td>
<td>2.1e-3</td>
<td>2.1e-3</td>
<td>(1.4e-3, 3.1e-3)</td>
</tr>
<tr>
<td>First-order rate constant for ER induction in liver (1/h)</td>
<td>k_ER,liv</td>
<td>Uniform (0.001, 0.1)</td>
<td>Assumed</td>
<td>0.082</td>
<td>0.083</td>
<td>(0.068, 0.096)</td>
</tr>
<tr>
<td>Inhibition constant for T production (1/h)</td>
<td>K_T</td>
<td>Log-uniform (0.001, 0.5)</td>
<td>Assumed</td>
<td>0.014</td>
<td>0.014</td>
<td>(8.8e-3, 0.020)</td>
</tr>
<tr>
<td>Concentration of microsomal protein in gonad (mg/l)</td>
<td>D_m np, gon</td>
<td>Log-uniform (3.1e +1, 3.1e + 5)</td>
<td>Measured by D. Villeneuve in female FHM</td>
<td>868</td>
<td>915</td>
<td>(76, 1.6e +3)</td>
</tr>
<tr>
<td>Concentration of microsomal protein in brain (mg/l)</td>
<td>D_m np, brn</td>
<td>Log-uniform (1.8e +2, 1.8e +6)</td>
<td>Measured by D. Villeneuve in female FHM</td>
<td>469</td>
<td>412</td>
<td>(198, 1.0e +3)</td>
</tr>
<tr>
<td>Scaling coefficient of V_max for Vtg production in liver (=V_max/bodyweight ^0.75) (nmol/h/kg^0.75)</td>
<td>sc_VmaxVtg, liv</td>
<td>Log-uniform (1, 1.0e +5)</td>
<td>Assumed</td>
<td>216</td>
<td>200</td>
<td>(103, 406)</td>
</tr>
<tr>
<td>Hill coefficient of Vtg production in liver</td>
<td>n_vtg</td>
<td>Log-uniform (2, 10)</td>
<td>Based on ER dimerization</td>
<td>3.2</td>
<td>3.2</td>
<td>(2.8, 3.7)</td>
</tr>
<tr>
<td>Partition coefficient of EE2 (blood to water)</td>
<td>λ_EE2, btd</td>
<td>Log-normal (600, 3)</td>
<td>Kim (2004)</td>
<td>283</td>
<td>238</td>
<td>(103, 740)</td>
</tr>
<tr>
<td>Partition coefficient of EE2 (liver to blood)</td>
<td>λ_EE2, liv</td>
<td>Log-uniform (1, 100)</td>
<td>Plowchalk and Teeguarden (2002)</td>
<td>2.8</td>
<td>2.5</td>
<td>(1.0, 6.7)</td>
</tr>
<tr>
<td>Scaling coefficient of V_max for KT production in gonad (=V_max/number of Leydig cells per gonad) (nmol/h/cell)</td>
<td>sc_Vmax 11bHSD, gon</td>
<td>Log-uniform (4.3e-9, 4.3e-6)</td>
<td>Ge et al. (1997)</td>
<td>5.3e-8</td>
<td>5.3e-8</td>
<td>(3.7e-8, 7.0e-8)</td>
</tr>
<tr>
<td>Variance of plasma E2 concentration in natural log-space</td>
<td>Var_Ln_CET2ot_pla_ngml</td>
<td>Inverse gamma (2, 0.56)</td>
<td>Bois et al. (1996a)</td>
<td>9.1</td>
<td>9.0</td>
<td>(7.3, 12)</td>
</tr>
<tr>
<td>Variance of plasma T concentration in natural log-space</td>
<td>Var_Ln_CItot_pla_ngml</td>
<td>Inverse gamma (2, 0.41)</td>
<td>Bois et al. (1996a)</td>
<td>0.31</td>
<td>0.30</td>
<td>(0.22, 0.42)</td>
</tr>
</tbody>
</table>

*Definition of prior distribution parameters P1 and P2. Log-normal: P1 = geometric mean (exponential of the mean in log-space); P2 = geometric standard deviation (exponential of the standard deviation in log-space, strictly superior to 1). Log-uniform: P1 = minimum of the sampling range (real number) in natural space; P2 = maximum of the sampling range in natural space. Uniform: P1 = minimum of the sampling range (real number) in natural space. P2 = maximum of the sampling range in natural space. Inverse gamma: P1 = shape; P2 = scale (both of the parameters are strictly positive).
natural logarithms of plasma E₂ concentrations used to calibrate the model is 15, and the posterior 95% CI is 7.3–12. In summary, model calibration with FHM data improved estimates of the majority of model parameters.

Model Evaluation

With the 4000 calibrated parameter sets, we simulated plasma concentrations of T, KT, E₂, and Vtg in unexposed male FHMs. Table 4 compares model predictions with plasma concentrations of T, KT, E₂, and Vtg measured in 73 unexposed (control) male FHMs; data that were not used to calibrate the model. Model-predicted 95% CIs for T, KT, and E₂ fall within the measured 95% CI for each endpoint. The model-predicted 95% CI for Vtg overlaps the measured 95% CI, but its lower bound is 80% that of the lower bound for the measured data. In unexposed FHMs, our model underpredicts the variance in the measured data.

We simulated plasma Vtg concentrations of male FHMs exposed to 10 and 100 ng EE₂/l reported in Ekman et al. (2008) (Fig. 3). Predicted plasma Vtg concentrations followed the same general trend as the measured concentrations. However, although the model predictions appear to be leveling-off by day 16, they do not start to decrease on day 16 like the measured concentrations. For the 10 ng EE₂/l exposure, the 95% CI of predicted Vtg concentrations encompassed the median concentration measured on all days. For the 100 ng EE₂/l exposure, the 95% upper confidence limit (or 97.5 percentile) of the simulated Vtg concentrations was 1.4–2 times lower than the median measured plasma Vtg concentration, except on day 16 where the median measured Vtg concentration fell within the 95% CI. These results suggest that the model is useful for predicting plasma Vtg responses for exposures up to 8 days, and up to 8 days postexposure.

Parks et al. (1999) reported that the plasma Vtg concentration on the seventh day after three injections of E₂ over a period of 4 days was 21.3 ± 1.3 mg/ml. We used our model and the 4000 calibrated parameter sets to simulate plasma Vtg concentrations (mg Vtg/ml) from Parks et al. (1999). Our simulations resulted in a mean = 29, standard deviation = 10 mg Vtg/ml, median = 27.5 mg Vtg/ml, and 95% CI = (14, 54). The predicted standard deviation in plasma Vtg concentration was larger than the measured data, and all of the measured data were within the 95% CI of the model predictions. In this case, our model predicts the mean measured plasma Vtg concentration well, but overpredicts the variance.

<table>
<thead>
<tr>
<th>Reproductive endpoint (plasma concentration)</th>
<th>Measured data</th>
<th>Model predictions</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (ng/ml)</td>
<td>13</td>
<td>8.6</td>
</tr>
<tr>
<td>KT (ng/ml)</td>
<td>37</td>
<td>35</td>
</tr>
<tr>
<td>E₂ (ng/ml)</td>
<td>0.38</td>
<td>0.20</td>
</tr>
<tr>
<td>Vtg (mg/ml)</td>
<td>0.0036</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

Note. Measured data include 73 male FHM. Model predictions include 4000 values generated using 4000 MCMC calibrated parameter sets.
Model Predictions of Unmeasured Endpoints in Male FHMs Exposed to EE2

As a demonstration of our model’s hypothesis generating capability, we used it to simulate 48-h exposures to four EE2 exposure concentrations: 5, 10, 50, and 100 ng EE2/l. The predictions shown in Figure 4 are a result of our model formulation and calibration, and experimental data to evaluate these predictions are needed. For the four different exposure concentrations, plasma concentrations of E2, T, KT, and Vtg as a function of time were predicted (Figs. 4A–D, respectively). Plasma concentrations of all three steroids are predicted to decrease as plasma EE2 concentration (Fig. 4E) increases. In contrast, plasma Vtg concentrations are predicted to increase as plasma EE2 concentration increases, and plasma Vtg concentrations remain elevated compared with unexposed FHM levels for at least 2400 h (100 days).

In addition to the measured reproductive endpoints, our model can also predict a number of model endpoints that, to date, have not been measured in FHMs such as plasma LH concentration (Fig. 4F), StAR protein concentration in gonads.
both data sets well, and concluded that these results are consistent. For example, Garcia-Reyero et al. (Forthcoming) exposed adult male FHMs to nominal concentrations of 10 and 50 ng EE2/l for 48 h, and measured plasma Vtg concentrations using a polyclonal FHM-based enzyme-linked immunosorbent assay (ELISA) (Hemming et al., 2001). Garcia-Reyero et al. (Forthcoming) reported plasma concentrations of 13 and 29 mg Vtg/ml, 48 h after the 10 and 50 ng EE2/l exposures, respectively. Ekman et al. (2008) exposed FHMs to nominal concentrations of 10 and 100 ng EE2/l for 8 days, and measured plasma Vtg using a polyclonal FHM-based ELISA (Parks et al., 1999). For the 10 and 100 ng EE2/l exposures, Ekman et al. (2008) reported the following plasma Vtg concentrations: on day 1, 1.2 and 8.7 mg Vtg/ml, respectively; on day 4, 28 and 62 mg Vtg/ml, respectively; and on day 8, 52 and 97 mg Vtg/ml, respectively. For the 10 ng EE2/l exposures from Garcia-Reyero et al. (Forthcoming) and Ekman et al. (2008) we found that our computational model fits both data sets well, and concluded that these results are consistent despite differences in measured water EE2 test concentrations, exposure duration, and Vtg analytical methods used in the two laboratories.

**DISCUSSION**

**Experimental Data**

At first glance, experimental data from different studies suggest a large amount of variability, possibly due to different laboratory conditions, exposure designs, chemical analysis methods, or FHM cultures. Although the data from different studies may appear variable, after experimental differences such as exposure design have been accounted for, the data become much more consistent. A reliable computational model can be used to evaluate whether data from different studies are in fact consistent. For example, Garcia-Reyero et al. (Forthcoming) exposed adult male FHMs to nominal concentrations of 10 and 50 ng EE2/l for 48 h, and measured plasma Vtg concentrations using a monoclonal carp-based enzyme-linked immunosorbent assay (ELISA) (Hemming et al., 2001). Garcia-Reyero et al. (Forthcoming) reported plasma concentrations of 13 and 29 mg Vtg/ml, 48 h after the 10 and 50 ng EE2/l exposures, respectively. Ekman et al. (2008) exposed FHMs to nominal concentrations of 10 and 100 ng EE2/l for 8 days, and measured plasma Vtg using a polyclonal FHM-based ELISA (Parks et al., 1999). For the 10 and 100 ng EE2/l exposures, Ekman et al. (2008) reported the following plasma Vtg concentrations: on day 1, 1.2 and 8.7 mg Vtg/ml, respectively; on day 4, 28 and 62 mg Vtg/ml, respectively; and on day 8, 52 and 97 mg Vtg/ml, respectively. For the 10 ng EE2/l exposures from Garcia-Reyero et al. (Forthcoming) and Ekman et al. (2008) we found that our computational model fits both data sets well, and concluded that these results are consistent despite differences in measured water EE2 test concentrations, exposure duration, and Vtg analytical methods used in the two laboratories.

**Model Structure and Formulation**

Many simplifying assumptions were made in formulating this computational model because many processes and parameter values are unknown for FHMs. There are too many to discuss them all in great detail, but in the following we discuss some of our assumptions that have alternative formulations.

In the brain, LH production is affected by E2, which is formulated in our model as a positive feedback based on studies in other fishes where T and E2 increased mRNA levels of LHβ subunit (Yaron et al., 2003). In addition, Yaron et al. (2003) describe an ERE in the promoter region of the Chinook salmon LHβ gene which suggests that bound ER (E2-ER or EE2-ER) would be a more realistic quantity than free E2 for positive feedback regulation of LH production. Such a modification to the model would allow all estrogenic chemicals (e.g., EE2) to stimulate LH production in brain.

In vertebrates, E2 production from T occurs in the brain and gonads catalyzed by the aromatase enzyme. Moreover, in teleost fishes, aromatase activity is relatively high in brain compared to gonads, and compared to brain aromatase in other vertebrate species (Callard et al., 2001; Sawyer et al., 2006), though E2 production rates depend on the number of cells in the respective tissues. Our model resulted in E2 production in brain at higher rates than in the testis. Calculations using fixed parameter values for BW (± 0.0041 kg), brain weight (± 3.8 × 10⁻⁵ kg), testis weight (± 5.5 × 10⁻⁵ kg), scaling coefficients of aromatase Vmax in brain and gonad (sc_Vmax_aro_brn = 1.05 × 10⁻² nmol/h/mg protein and sc_Vmax_aro_gon = 2.3 × 10⁻³ nmol/h/mg protein, respectively), and the median calibrated concentrations of microsomal protein in brain (D_m_mp_brn = 500 mg protein/l) and gonad (D_m_mp_gon = 800 mg protein/l), we found E2 production in brain to be twice as high as E2 production in testis, 2.0 × 10⁻⁴ nmol/h and 1.0 × 10⁻⁴ nmol/h, respectively. This is consistent with studies in teleost fish that describe higher aromatase activity in brain compared to gonads (Callard et al., 2001; Halm et al., 2001; Sawyer et al., 2006).

**Model Calibration**

The posterior parameter distributions obtained through model calibration depend upon values of the fixed parameters (Supp. Info. Table S1), the prior parameter distributions (Table 3), and the measured reproductive endpoints. In developing our model, we relied upon values based on data from other species and some in vitro studies because of the lack of FHM-specific data. It is important to note that FHMs (e.g., zebrafish, or Japanese medaka) are a small aquarium-sized fish and an asynchronous spawner (i.e., multiple spawns in a season with eggs present in all stages of maturation simultaneously in the gonads) as opposed to synchronous spawners (e.g., salmon, rainbow trout, or channel catfish) in which the eggs more or less mature synchronously during a spawning season. Conditional upon the fixed model parameter values, we believe that our model and the calibrated model parameters provide a good representation of the HPG axis in FHMs. If FHM-specific values of our fixed model parameters were appreciably different from what we used in this modeling study, then the calibrated model parameters would probably have different values, but the physiologically based model as formulated could perform as well as shown here or better.

**Model Evaluation**

We evaluated our model’s predictive ability by simulating EE2 and E2 laboratory experiments in FHMs from two studies...
In each of these studies, the exposure pattern of EE$_2$ or E$_2$ differed from the exposure patterns used to obtain the model calibration data. It is noteworthy that the model was able to accurately predict plasma Vtg concentrations after an 8-day continuous exposure to a nominal concentration of 10 ng EE$_2$/L from Ekman et al. (2008) (Fig. 3A). The model underpredicted all plasma Vtg concentrations in FHM exposed to a nominal water concentration of 100 ng EE$_2$/L, but this exposure concentration is higher than either of the 48-hr exposure concentrations used to calibrate the model, and is therefore an extrapolation of the model’s capability. For the Parks et al. (1999) study, three injections of E$_2$ were given at a higher dose than either of those used to calibrate the model. Thus, it is remarkable that the model-predicted plasma Vtg concentrations encompass the measured data. These results suggest that our model has captured the processes important for steroid hormone and Vtg production in male FHMs.

A limitation of our model is that it significantly underpredicts variance in measured plasma steroid hormone and Vtg concentrations for unexposed fish (Table 4), though it predicts median concentrations well. Interestingly, this is not the case for exposed fish. Indeed, the model tends to slightly overpredict variance in measured plasma Vtg concentrations for fish exposed to EE$_2$ from Ekman et al. (2008) (Fig. 3). Furthermore, the model significantly overpredicts variance in measured plasma Vtg for fish exposed to E$_2$ from the Parks et al. (1999) study.

Perhaps this tendency of the model to underpredict variance in unexposed fish, and to overpredict variance in exposed fish is not surprising, given the nature of interindividual variation that is often observed in molecular profiling studies for chemical exposures. Namely, researchers often find that interindividual variation in measured values for controls is larger than that for exposed animals (Parsons et al., 2009). For example, using $^1$H NMR spectroscopy for hepatic metabolite profiling, unexposed female fathead minnows were found to exhibit considerably more variation than females that were exposed to estrogenic compounds (Ekman et al., 2006). This was attributed to unexposed fish being in different stages of the reproductive cycle. However, upon exposure, the reproductive status of the female fish was more homogeneous, resulting in a more “focused” metabolic response. Beyond this specific example using female fish, this effect of focusing upon exposure appears to be quite common regardless of animal, gender or tissue/fluid type (Parsons et al., 2009).

Indeed, we see a striking example of this focusing effect when examining lipid metabolite profiles of plasma samples from the same male FHMs used to evaluate this model (i.e., fish from Ekman et al., 2008). Figure 5 is a principle components analysis scores plot built from NMR spectra of the pool of plasma lipid metabolites from male FHMs measured by $^1$H NMR spectroscopy. PC1 = principal component 1; PC2 = principal component 2.
characterized by each set of 20 calibrated model parameters) that are represented by the measured data. Changes in reproductive endpoints as they relate to the “population” can then be assessed for new exposure conditions. Furthermore, with the appropriate modifications and initial conditions (e.g., plasma T, KT, and E2 concentrations at birth), our model could be extended to simulate FHM early life stages.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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