Materials and Methods

Animals

Serum from animals that were exposed during gestation or lactation was used for thyroid hormone analysis. Specifics regarding animal care, use, and exposure are described in detail by Phillips et al. [1].

Serum Thyroid Hormone Analysis

Unlabeled thyroxine (T4), triiodothyronine (T3), reverse triiodothyronine (rT3), citric acid, ascorbic acid, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO). Internal standards, $^{13}C_{12}$-T4, $^{13}C_6$-rT3, and $^{13}C_6$-T3, and the recovery standard, $^{13}C_6$-T4, were purchased from Cambridge Isotope Laboratories (Andover, MA). Both the extraction and analysis step were monitored with respect to recovery and process efficiency by using these two different stable isotope T4 internal standards.
All solvents used were HPLC grade (EMD Millipore Corporation, Bellerica, MA).

Chemical Analyses

Concentrationss of total thyroxine (TT4), total triiodothyronine (TT3) and total reverse triiodothyronine (TrT3) were quantified in the serum of dams at GD 18 and PND 12 and in the serum of pups at PND 12. Due to low volumes, serum was pooled among pups of the same sex from each litter. Extraction of thyroid hormones from rat serum was performed according to a previously published method (1). Briefly, labeled internal standards $^{13}C_{12}$-T4, $^{13}C_6$-rT3, and $^{13}C_6$-T3 were added to each serum sample to quantify the concentrations of T4, rT3, and T3, respectively. An antioxidant/reducing solution containing 25 g/L ascorbic acid, citric acid, and DTT was added and serum samples were equilibrated on ice for 1 hour. Hydrochloric acid was then added and samples were incubated in an oscillating water bath held at 50°C for 1 hour. Serum samples were cleaned using SampliQ OPT SPE cartridges (30 mg, 1 mL, Agilent). Analytes were eluted using 4 mL of methanol, blown down to near dryness, and reconstituted with 1:1 methanol/water (v/v). Prior to analysis, $^{13}C_6$-T4 was added as a recovery standard and samples were transferred to a Mini-Uniprep Syringeless Filter (PTFE, 0.2 μm; Whatman/GE Healthcare).

Statistical Analysis

Thyroid hormone statistical analyses were performed using StatView® software (version 5.0.1; SAS Institute Inc., Cary, NC) and statistical significance was set at $\alpha = 0.05$. One T3 value from the high dose lactational dam group was identified as an outlier (greater than two standard deviations from the mean) and removed from the dataset. The lowest T3 value from the high dose lactational dam group was also removed to produce a trimmed mean as recommended by [2]. No other outliers were removed from any of the datasets and data were not log transformed for any of the analyses. ANOVA was used to assess the effects of FM 550 dose on serum TT3 and TT4 concentrations in both gestationally and lactationally exposed dams, separately. As in Phillips *et al.*, a repeated-measures ANOVA was performed with sex being considered a repeated measure and the litter as the definition of n =1 to evaluate the effects of sex and FM 550 dose on pup serum TT3 and TT4 levels [1]. The sample size, *n*, of each group is shown in Table 1. Thyroid data within the text are reported as mean ± SEM.

Results: Thyroid Hormone Concentrations

Levels of total thyroxine (TT4), total triiodothyronine (TT3) and total reverse triiodothyronine (TrT3) were quantified in the serum of dams at GD 18 and PND 21 and in the serum of pups at PND 21. Average recovery of internal standards were as follows: 65.6 ± 6.2% for $^{13}C_{12}$-T4, 75.6 ± 14.9% for $^{13}C_6$-rT3, and 61.0 ± 5.3% for $^{13}C_6$-T3. Method detection limits (MDLs) were 6.1 ng/mL for TT4, 0.6 ng/mL for rT3, and 0.2 ng/mL for TT3. TrT3 was not detected above the MDL in any of the serum samples. Average TT4 and TT3 serum concentrations (ng/mL) are shown in Table 2. Compared to lactating controls, TT3 concentration in the serum of lactating dams in the low dose group were significantly elevated ($p = 0.557$).