Maternal diet composition alters serum steroid and free fatty acid concentrations and vaginal pH in mice

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Abstract

We examined the effects of three maternal diets (very high fat (VHF), low fat (LF), and control (Purina 5015)) on serum steroids, free fatty acids (FFA), and vaginal pH in National Institutes of Health Swiss mice. Females were fed (VHF, $n=33$; LF, $n=33$; 5015, $n=48$) from 4 to 16 weeks of age. Following breeding, female serum was collected at 0.5 (pre-implantation, early diestrus) or 8.5 (post-implantation, mid-diestrus) days post-coitus (dpc). The serum concentrations of 17 β -estradiol, testosterone, progesterone, and FFA were analyzed at both collection points, and vaginal pH at 0.5 dpc. Striking differences in steroids and FFA were observed at 0.5 dpc among the groups. Estradiol was higher in the VHF (14.1 ± 3.0 pg/ml), compared with LF mice (5.2 ± 2.3 pg/ml; $P \leq 0.05$). In contrast, 0.5 dpc testosterone was lower in the VHF (10.5 ± 3.0 pg/ml) versus the LF group

(32.7 ± 8.4 pg/ml; $P \leq 0.05$). At 8.5 dpc, progesterone was higher in the VHF (89.6 ± 6.7 ng/ml) versus the 5015 group (60.1 ± 4.9 ng/ml; $P \leq 0.05$). VHF mice had higher FFA concentrations at 0.5 dpc (1.0 ± 0.2 mmol/l) than LF and control mice (0.5 ± 0.1 and 0.6 ± 0.1 mmol/l respectively; $P \leq 0.05$). At 8.5 dpc, VHF females had higher serum FFA (0.8 ± 0.1 mmol/l) than LF and control females (0.4 ± 0.1 and 0.6 ± 0.1 mmol/l; $P \leq 0.05$). Mean vaginal pH of VHF females (6.41 ± 0.09) was lower than 5015 females (6.76 ± 0.10 ; $P \leq 0.05$). These diet-induced alterations in serum steroid and FFA concentrations might affect several reproductive processes, including preferential fertilization by one class of sperm over the other and sex bias in pre- and post-implantational embryonic development.

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Introduction

For several years, this laboratory has been interested in the effects maternal diet can have on offspring sex ratio [Rosenfeld et al. 2003](#). Many past and present reports have suggested that diet, particularly one enriched with either saturated or unsaturated fatty acids, can alter serum steroid concentrations in a variety of species, including rodents, food animals, and humans ([Talavera et al. 1985](#), [Hilakivi-Clarke et al. 1996](#), [Woods et al. 1996](#), [Hilakivi-Clarke et al. 1997](#), [Dorgan et al. 2003](#)). Thus, as a potential aside, we were interested in examining the effects of three diets: very high fat/low fat (VHF, LF; Research Diets, New Brunswick, NJ, USA), and Purina 5015 chow-based diet (Purina, St Louis, MO, USA; see [Table 1](#)) on serum steroid concentrations and free fatty acids. While the mechanisms underlying diet-induced alteration of offspring sex ratio are likely complex, any diet-induced differences in steroid concentration may help to elucidate how diet skews offspring sex ratio.

Dietary fat can influence the expression of enzymes that metabolize sex steroid hormones ([Zhou et al. 2005](#), [Dieudonne et al. 2006](#)). Adipose tissue is an important site of steroid hormone biosynthesis ([Siiteri 1987](#), [Belanger et al. 2002](#), [Simpson 2003](#), [Dieudonne et al. 2006](#)). Moreover, ovarian-derived $\Delta 4$ androstenedione and testosterone can be aromatized

in adipose tissue to estrone and estradiol respectively ([Lambrinoudaki et al. 2006](#)). An additional potential mechanism of dietary influence on sex steroid concentration relates to the status of cholesterol as a precursor to steroid hormones. Diet can influence serum cholesterol ([Menotti 1999](#)) and high cholesterol is correlated with high serum androgen and estrogen concentrations ([Shelley et al. 1998](#), [Kumagai et al. 2001](#)). Past studies in rodents, cattle, and humans have indicated that diet might underpin changes in serum hormonal concentrations, including testosterone and estrogen ([Killen et al. 1989](#), [Wynn & Wynn 1993](#), [Dorgan et al. 1996](#), [Hilakivi-Clarke et al. 2002](#), [Dorgan et al. 2003](#), [Fernandez-Twinn et al. 2003](#)). Female rats fed a diet enriched with n-3 polyunsaturated fatty acids had a 48% increase in serum concentrations of 17 β -estradiol compared with rats fed a diet enriched with n-6 fatty acids ([Hilakivi-Clarke et al. 2002](#)). Similarly, female rats fed a low protein diet had a significant increase in 17 β -estradiol compared with those fed a control diet ([Fernandez-Twinn et al. 2003](#)). Nutritionally restricted heifers have decreased serum estrogen concentrations at day 200 of pregnancy compared with larger, non-food restricted heifers ([Killen et al. 1989](#)). A high saturated fat diet induces an increase in estrogen, estrone, and dehydroepiandrosterone sulfate concentrations in women ([Dorgan et al. 1996](#), [Nagata et al.](#)

2005). A controlled clinical trial revealed that girls fed a low fat (LF) diet exhibited higher serum testosterone concentrations during the luteal phase of the cycle but lower estradiol concentrations (Dorgan *et al.* 2003).

In the light of these previous studies, we have examined the serum concentrations of testosterone, estrogen, progesterone, and free fatty acids (FFA) in female mice fed a predominantly lard diet (which skews the offspring sex ratio to males), a LF/high carbohydrate diet (which skews the ratio towards females), and a chow-based control diet (which gives rise to roughly equivalent numbers of male and female pups). Our underlying hypothesis was that diet might alter various serum steroid concentrations, which in turn may influence the reproductive tract environment, such as vaginal pH, at copulation or during embryonic development to favor the success of one sex over the other.

Materials and Methods

Animal breeding and diet conditions

NIH Swiss mice (*Mus musculus*) were bred and maintained in the University of Missouri's Animal Science Research Center. All the experiments were carried out according to the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Missouri's Animal Care and Use Committee. Mice were housed in polysulfone cages (Uni Cage, Siloam Springs, AR, USA; dimensions: 18.4 cm W × 29.2 cm D × 12.7 cm H). During non-breeding periods, four female mice were housed per cage, and during breeding, two females were housed with one male.

All mice used in this study were NIH Swiss and were bred and assigned to diet groups as described previously (Rosenfeld *et al.* 2003). In December 2004 (Study 1), 8 to 10-week-old P1 NIH Swiss female mice (Harlan, Madison, WI, USA) were bred to stud males. After weaning at 21 days, 18 female offspring were maintained on a regular chow diet, Purina 5015 (5015), until they were 30 days of age and then randomly assigned to three groups and fed *ad libitum* the control Purina 5015 ($n=10$), low fat (LF; $n=4$), or very high fat (VHF; $n=4$) diets throughout the study. The females were bred at 20 weeks of age to stud NIH Swiss males (≈ 12 -week-old). A replicate study was initiated 2 weeks after beginning the first to provide a total of 13 mothers each in the LF and VHF dietary groups and 28 mothers in the 5015 dietary group. To increase the sample size in each diet group, two additional experiments of identical design were initiated in October 2005 (Study 2; $n=6$ females per group, 16 weeks on diets) and December 2005 (Study 3; $n=14$ females per group, 16 weeks on diets) to provide an overall total of 33 females in the LF and VHF diet groups, and 48 females in the 5015 diet groups.

Conception was assessed by the presence of a coital plug (the day of the coital plug being scored as 0.5 days post-coitus (dpc)), and females were assessed to be pregnant by the presence of either pre-implantational embryos in the oviduct (0.5 dpc) or implanted conceptuses in the uterine wall (8.5 dpc). Vaginal pH was determined at 0.5 dpc in all females as described in the next

section. Females were then randomly assigned to one out of the two study groups. In the first group, pregnant females (VHF; $n=15$; LF; $n=14$; 5015, $n=23$) were killed after pH determination by CO₂ inhalation and subsequent cervical dislocation. Collection of serum for steroid hormone and FFA concentrations was performed immediately. In the second group (VHF; $n=11$; LF; $n=17$; 5015, $n=17$), vaginal pH was determined, and females were housed in separate cages and maintained on their assigned diet until 8.5 dpc. At this point, females were killed for serum collection as described above.

Vaginal pH determination

Vaginal pH measurements were performed by using an MI-413 Needle Combination pH microelectrode (Microelectrodes Inc., Bedford, NH, USA) with electrode diameter of 16 G needle and BASIC pH meter (Denver Instruments, Denver, Colorado, USA). Mice were immobilized in a restrainer made from a 50 ml Falcon centrifuge tube (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Each mouse was placed inside the tube and fixed with the cap from the same tube with 15 mm diameter hole in it, thereby allowing access to the tail and the vaginal opening. Presented data are averages from at least five independent measurements on each mouse.

Blood collection and serum preparation

A non-heparinized whole blood sample was obtained by cardiac puncture, and the microfuge tubes containing the whole blood were placed on ice for 30 min to permit clotting. The samples were centrifuged at 7500 *g* for 20 min. The resulting serum fraction was collected and transferred to a new autoclaved microcentrifuge tube and centrifuged at 7500 *g* for 3 min to remove further traces of clotted blood. Aliquots of serum for 17 β -estradiol, progesterone, and testosterone enzyme immunoassay (EIA) were stored at -20°C , and aliquots of serum for FFA determination were stored at -80°C .

Estradiol assays

Circulating serum concentrations of 17 β -estradiol were analyzed by using a modified EIA procedure initially described by Perry *et al.* (2004). Duplicate samples (50 μl) were extracted with 2 ml methyl-*tert*-butyl ether (HPLC grade; Fisher Chemical Co., Fair Lawn, NJ, USA) for 2 min on a vortexer. Samples were frozen in a dry ice-methanol bath. The solvent fraction was decanted into 12 × 75 mm borosilicate glass tubes and dried under a stream of nitrogen gas. Extracts were reconstituted in 50 μl estradiol-free control serum.

Estradiol EIA kits and reagents were obtained from Diagnostic Systems Laboratories, Inc. (DSL; Webster, TX, USA) and Cayman Chemical (Ann Arbor, MI, USA). The assays were performed according to the manufacturer's instructions. All extracted samples were run in duplicate on a 96-well plate and measured on a Bio-Tek Synergy HT plate reader at 450 (DSL) or 412 nm (Cayman) absorbance (Bio-Tek

Instruments Inc., Winooski, VT, USA). Estradiol concentrations in samples were determined based on a 17β -estradiol standard curve (DSL: 0–2000 pg/ml; $r^2=0.98$; Cayman: 7.8–1000 pg/ml; $r^2=0.97$). Concentrations of 17β -estradiol in two duplicate control standards (250 ± 100 and 1000 ± 350 pg/ml; DSL) were determined within the specified margin of error. Intra- and inter-assay coefficients of variation for the Cayman 17β -estradiol assay were 3.3 and 7.6% respectively. Intra- and inter-assay coefficients of variation for the DSL 17β -estradiol assay were 4.6 and 11.2% respectively.

Progesterone and testosterone assays

Concentrations of free progesterone and testosterone were determined by EIA with unextracted serum samples. Progesterone and testosterone EIA kits and reagents were obtained from Diagnostic Systems Laboratories, Inc. Each assay was run according to the manufacturer's protocols. All the serum samples (50 μ l) were run in duplicate and measured on a Bio-Tek Synergy HT plate reader at 450 nm absorbance (Bio-Tek Instruments Inc.). Serum progesterone concentrations were determined based on a five-point progesterone standard curve (0.3–80 ng/ml; $r^2=0.99$). Concentrations of progesterone in two duplicate control standards (1 ± 0.3 and 7 ± 2 ng/ml; DSL) were determined within the specified margin of error. Testosterone concentrations in samples were determined based on a six-point testosterone standard curve (5–3000 pg/ml; $r^2=0.99$). Concentrations of testosterone in two duplicate control standards (50 ± 15 and 250 ± 50 pg/ml; DSL) were determined within the specified margin of error. Intra- and inter-assay coefficients of variation for the testosterone assay were 3.0 and 10.5% respectively. Intra- and inter-assay coefficients of variation for the progesterone assay were 3.7 and 12.6% respectively.

Serum free fatty acids

Concentrations of FFA were measured in triplicate using an enzymatic assay and according to the manufacturer's instructions (Wako Chemicals, Richmond, VA, USA) and other published methods (Parks *et al.* 2005). Concentrations of FFA were determined based on a four-point FFA standard curve (0–1 mmol/l; $r^2=0.99$). The intra- and inter-assay coefficients of variation were 16.9 and 9.9% respectively.

Statistical analyses

The results are expressed as the mean $\pm$ S.E.M. Effects of diet on serum steroids and FFA were examined by ANOVA as a 2×3 factorial arrangement with time of serum collection and diet as factors respectively. Effect of diet on vaginal pH was tested by using one-way ANOVA. Distributions of continuous variables were tested for normality by use of the Kolmogorov–Smirnov test. Endpoints with heterogeneous variance were logarithmically transformed. *Post hoc* treatment effects were tested by using Fisher's least significant difference mean separation test. Pearson's

product moment correlation was employed to determine the relationship between serum steroid concentrations and maternal weight and embryo number. The n used for the analyses varied for the different analyses because for some samples there was not enough serum to perform all of the EIA for the steroids and the FFA. Statistical significance was set at the 0.05 level (SAS 1988).

Results

Vaginal pH

At 0.5 dpc, female NIH Swiss mice in the VHF diet group had significantly lower vaginal pH (6.41 ± 0.09 ; $n=16$) compared with the 5015 control group (6.76 ± 0.10 ; $n=19$; ANOVA, $P \leq 0.05$). However, this statistical difference did not extend to the LF (6.61 ± 0.08 ; $n=19$) diet group.

Maternal body weights

As was observed in a previous study (Rosenfeld *et al.* 2003), the mice tolerated the different diet conditions well, including the high lard content of the VHF diet. Maternal body weights over the 16-week dietary treatment period are displayed in Fig. 1. The greatest increase in body weight over time was seen in VHF-fed females, but this difference was only significant in Study 3 (Table 1; ANOVA; $P \leq 0.05$). Despite the lack of maternal body weight difference among diet treatments, in general, body composition was visibly different during dissection, with VHF-fed females having larger amounts of visceral fat.

Serum estradiol

Mean concentrations of extracted serum estradiol at 0.5 dpc in mice fed the VHF diet (14.1 ± 3.0 pg/ml; Fig. 2) were significantly higher than mice fed the LF (5.2 ± 2.3 pg/ml; $P \leq 0.05$) and 5015 control (8.2 ± 2.3 pg/ml; $P \leq 0.05$) diets. At 8.5 dpc, the relative concentrations of extracted serum estradiol in mice-fed VHF and LF diets appeared to be inverted compared with the 0.5 dpc means, but did not differ statistically at this collection time point (Fig. 2). Extracted serum estradiol at 0.5 dpc was correlated with breeding maternal body weight (g) when the three diet groups for all the study times were analyzed ($r=0.55$; $n=44$; $P \leq 0.05$). No correlation with serum estradiol was detected for breeding maternal body weight at 8.5 dpc.

Serum testosterone

Mice fed a LF diet had significantly higher mean serum testosterone concentrations at 0.5 dpc (32.7 ± 8.4 pg/ml) compared with both VHF (10.5 ± 3.0 pg/ml) and 5015 control (12.9 ± 3.4 pg/ml)-fed mice (Fig. 2; ANOVA; $P \leq 0.05$). In contrast, at 8.5 dpc (Fig. 2), VHF-fed mice had significantly higher serum testosterone concentrations (77.8 ± 27.8 pg/ml) compared with 5015 control-fed mice (51.3 ± 40.7 pg/ml; ANOVA; $P \leq 0.05$). However,

Figure 1 Changes in mean maternal body weight ($\pm$ S.E.M.) of mice until breeding on the VHF ($\blacktriangle$; $n=23$), LF ($\blacksquare$; $n=28$), and 5015 control ($\blacklozenge$; $n=35$) diets. The data are from three studies, with females maintained on diets for 16 weeks starting at 30 days of age. Significant weight difference of VHF females from LF and control groups is denoted by asterisks (ANOVA; $P<0.05$).

testosterone concentrations in LF-fed mice at 8.5 dpc (62.3 ± 27.7 pg/ml) did not differ from the other two groups. As was the case with serum estradiol, serum testosterone at 0.5 dpc was negatively correlated with breeding maternal body weight (g) when the three dietary groups for all study times were analyzed ($r = -0.38$; $n=43$; $P \leq 0.05$). No relationship existed between 8.5 dpc serum testosterone concentration and maternal breeding weight.

Serum estradiol/testosterone (E/T) ratio

The ratio of extracted serum estradiol to serum testosterone in individual mice was on average greater than one for females on the VHF diet at 0.5 dpc (23.3 ± 11.6 ; $n=12$) and less than

Table 1 Effect of control (5015), LF, and VHF diets on embryo number and maternal weight (g) of female mice aged 20 weeks at breeding (mean $\pm$ S.E.M.)

	Diet	Maternal weight (g; 20 weeks)
Breeding date		
	5015	29.5 $\pm$ 0.7 ($n=22$)
	LF	29.1 $\pm$ 0.9 ($n=10$)
Study 1 (July 2005)	VHF	32.5 $\pm$ 1.4 ($n=9$)
	5015	29.3 $\pm$ 3.6 ($n=4$)
	LF	26.8 $\pm$ 0.7 ($n=4$)
Study 2 (February 2006)	VHF	32.5 $\pm$ 4.0 ($n=4$)
	5015	34.2 $\pm$ 5.6 ($n=9$) ^a
	LF	30.0 $\pm$ 2.2 ($n=14$) ^a
Study 3 (April 2006)	VHF	47.0 $\pm$ 11.8 ($n=10$)
	5015	30.7 $\pm$ 0.7 ($n=35$) ^a
	LF	29.2 $\pm$ 0.5 ($n=28$) ^a
Combined	VHF	38.6 $\pm$ 2.3 ($n=23$)

Three identical study designs conducted on different dates are analyzed separately and in combination. Embryo number counts include both 0.5 and 8.5 dpc embryos collected. Lowercase letters denote significant differences among dietary treatments (ANOVA; $P \leq 0.05$).

one for females on the LF diet at 0.5 dpc (0.2 ± 0.1 ; $n=9$). The mean LF female E/T ratio was significantly lower than both the VHF and the 5015 ratios (ANOVA; $P \leq 0.05$) at 0.5 dpc. The mean E/T ratios for the three dietary treatments at 8.5 dpc differed significantly (ANOVA; $P \leq 0.05$), but in contrast to the 0.5 dpc results, the E/T ratio in individual mice was generally less than one for females on the VHF diet (0.2 ± 0.1 ; $n=8$) and greater than one for females on the LF diet (5.5 ± 3.8 ; $n=11$). At 0.5 and 8.5 dpc, mean E/T ratios for mice on the 5015 control diets were 3.2 ± 1.7 ($n=17$) and 1.7 ± 0.6 ($n=12$) respectively.

Serum progesterone

Serum progesterone at 0.5 dpc did not differ among the dietary groups (Fig. 2), but the concentration of this steroid was inversely related to maternal body weight at the time of breeding ($r = -0.36$, $n=44$, $P \leq 0.05$). At 8.5 dpc, (Fig. 2), VHF-fed mice had significantly higher serum progesterone concentrations (89.6 ± 6.7 ng/ml) compared with 5015 control-fed mice (60.1 ± 4.9 ng/ml; ANOVA; $P \leq 0.05$). In contrast to the negative correlation at 0.5 dpc, a significant positive relationship existed between maternal body weight at the time of breeding and serum progesterone concentration at 8.5 dpc ($r = 0.49$, $n=34$, $P \leq 0.05$).

Serum free fatty acids (FFA)

Mice in the VHF diet group had an elevated mean concentration of FFA at 0.5 dpc (Fig. 2; 1.0 ± 0.2 mmol/l) compared with both the LF and the 5015 control diet groups (0.5 ± 0.1 and 0.6 ± 0.0 respectively; ANOVA; $P \leq 0.05$). This same pattern of concentrations was observed in the different dietary groups at 8.5 dpc, with significant differences among all the three groups (Fig. 2; ANOVA; $P \leq 0.05$). No relationship between FFA and maternal body weight at breeding was observed at either 0.5 or 8.5 dpc ($n=48$ and 37 respectively).

Discussion

Herein, we have examined the influence of diet on some reproductive variables in mice, including serum concentrations of sex steroids and vaginal pH, and discovered that diet can influence these parameters. The interactions between diet and circulating steroid hormone concentrations were complex. The VHF diet increased serum estrogen concentrations relative to the other dietary treatments at 0.5 dpc, but not at 8.5 dpc. Previous studies have shown that serum estrogen concentrations are highest at proestrus and decline during pregnancy (McCormack & Greenwald 1974, Nelson *et al.* 1992, Walmer *et al.* 1992; reviewed in Overpeck *et al.* 1978). The higher amount of estradiol in the mice on the VHF diet compared with the other diet groups could be related to the amount of accumulated body fat in the mice.

Figure 2 Steroid hormone concentrations (estradiol, testosterone, and progesterone) and free fatty acid (FFA) concentrations at 0.5 (black bars) and 8.5 dpc (open bars) in serum of female NIH Swiss mice maintained on very high fat (VHF), low fat (LF), and control (5015) diets for 16 weeks. Lowercase letters denote significant differences among dietary treatments within the 0.5 and 8.5 dpc results ($P \leq 0.05$) for each endpoint. Female sample size is denoted within each bar and the mean concentration for each group is denoted above each bar.

Adipose tissue is the major tissue site of conversion of androstenedione to estrone, and estradiol has been reported to be elevated in obese humans and non-human primates (reviewed in Siiteri 1987, Dieudonne *et al.* 2006). Maternal body weight was higher overall in the VHF mice in this study (Fig. 1) and an earlier study (Rosenfeld *et al.* 2003), and the extra visceral fat in these mice probably provided an additional estrogen source. Analogous dietary interventions exert similar effects on circulating estrogen concentrations. Rats fed a diet enriched with $n-3$ FFA had higher estrogen concentrations than their counterparts fed a normal, non-supplemented diet (Hilakivi-Clarke *et al.* 2002, Fernandez- Twinn *et al.* 2003). Conversely, nutritionally restricted cattle had lower estrogen concentrations at day 200 of pregnancy than controls (Killen *et al.* 1989). A meta-analysis of studies in women suggested that increased dietary fat intake resulted in elevated serum estradiol concentrations (Wu *et al.* 1999, 2000). Finally, Dorgan *et al.* (2003) noted that a 5-year reduction in dietary

fat in pre-pubertal girls reduced serum estradiol (measured at the follicular phase) but increased serum testosterone concentrations (measured at the luteal phase). Whether the amount of accumulated body fat is the main variable in the above studies is unclear. As reviewed by Lambrinoudaki *et al.* (2006), there have been conflicting reports in women as to whether high serum estrogens are positively correlated with circulating cholesterol and triglyceride concentrations. Species variation and interaction of diet and timing of the estrous or menstrual cycle might account for these seemingly disparate results.

At 0.5 dpc, the three groups (VHF, LF, and 5015 controls) had very different E/T ratios. The VHF group, in particular, had relatively high estradiol values and somewhat reduced testosterone values relative to the 5015 controls, whereas the LF-fed mice had elevated circulating testosterone concentrations. Clearly, the relative concentration of the two sex steroids is affected by diet. As discussed above, the low

testosterone and high estradiol concentrations in VHF females at 0.5 dpc may reflect an increased conversion of androgen to estrogen in adipose tissue. The relatively higher testosterone concentrations in the LF-fed females at 0.5 dpc is seemingly harder to explain but is consistent with Dorgan *et al.* (2003) findings that girls fed a reduced calorie diet had higher testosterone concentrations, suggesting that a reduced adipose tissue mass results in a buildup of androgen. In serum collected at 8.5 dpc, the *E/T* relationship between VHF and LF groups was reversed, in large part because of the increased concentration of testosterone and the reduction of estradiol in the VHF-fed mice. The excess of circulating cholesterol, as would be anticipated in mice on a diet rich in saturated fat, might account for the relatively high concentrations of progesterone and its downstream metabolite, testosterone (Arensburg *et al.* 1999) in the VHF group at 8.5 dpc.

It remains to be determined whether these differences in steroid concentrations could influence the sex ratio of pups. Male fetuses have been proposed to be more sensitive to progesterone insufficiency than females (Flint *et al.* 1997). However, the concentration of this hormone did not differ significantly between the VHF and the LF groups at either 0.5 or 8.5 dpc. Moreover, values in the LF group at 8.5 dpc were not different from those measured in the controls. Clearly, progesterone is an unlikely mediator of sex ratio skewing in this case.

Androgens are known to exert epigenetic effects on the developing fetus. For example, female mice born between two males (2M) tend to be masculinized and have more male offspring (Vandenbergh & Huggett 1995, Vom Saal *et al.* 1999). Dominant women with elevated serum testosterone concentrations are reported to have significantly more sons than submissive females with lower testosterone concentrations (Grant 1994, Singh & Zambarano 1997, Grant & France 2001), and James (1990) has implicated high testosterone concentrations in the mother around the time of conception as a factor likely to favor sons. However, our data show that the mice on the LF diet unexpectedly had the highest testosterone concentrations at 0.5 dpc and had roughly similar concentrations to the VHF group at 8.5 dpc. Since mature LF females produce more female than male pups, testosterone seems to be an unlikely mediator of the skewing towards male pups in mice on the VHF diet.

Another difference between mice on the VHF and the LF diets was the concentration of circulating FFA. At 8.5 dpc, in particular, serum FFA was higher in the VHF group, a perhaps not surprising outcome, considering that these mice were consuming almost sixfold more calories from fat than the LF group. Little is known about the effects of FFA on the developing embryo and whether there is any sexual dimorphism with regard to either their utilization or relative toxicity. Nor is it clear whether FFA could influence the properties of X and Y sperm in some selective manner. The lower vaginal pH of mice on the VHF diet might be an outcome of their higher circulating concentrations of FFA or estrogen (Gorodeski *et al.* 2005). Moreover, the motility of X

and Y sperm may be influenced by vaginal pH (Ericsson 1976, Pratt *et al.* 1987), with more acid conditions providing an advantage to Y-bearing sperm. In hamsters, more male births occur if fertilization occurs late in estrus, possibly as the result of low vaginal pH (Pratt *et al.* 1987).

Studies in opossums and humans have implicated elevated dietary FFA, and particularly the essential polyunsaturated fatty acids (PUFA), in increasing male births (Austad & Sunquist 1986). The presence of excessive lard in the VHF diet, which leads to more male births, could conceivably provide a sparing effect on metabolism of PUFA by mice in the VHF diet group, if indeed PUFA are important in controlling sex ratio, e.g. by altering the production of reproductively significant prostaglandins (PGs), such as PGE or PGF_{2α} (Thatcher *et al.* 1995). To examine the effects of PUFA, we are beginning a study to determine whether diets differing in PUFA but equivalent in total fat calorie content are able to cause a change in sex ratio among pups born to NIH Swiss mice.

To determine whether, indeed, a potential linkage exists between steroid concentrations at the time of conception and alteration of sex ratio, we collected all of the 1-cell embryos at 0.5 dpc from the mice in the third study ($n=14$ dams on the LF diet and $n=10$ for dams on the VHF diet). All of their embryos were cultured to the blastocyst stage and the gender of each was determined by XY-FISH analysis with probes from Open Biosystems (Huntsville, AL, USA). The preliminary results from this study suggest that a sex ratio skewing is already present at 0.5 dpc with an increased ratio of male blastocysts in the VHF group and more female blastocysts in the LF group, as determined by the GENMOD procedure in SAS and logit transformation (odds ratio) ($P \leq 0.01$). Further studies will need to be performed to confirm these preliminary results and determine whether the differences in the sex steroids and FFA concentrations at this time point in gestation underpin this putative skewing in sex ratio.

In summary, the amount of fat in the diet fed to female mice, particularly around the time of early diestrus (conception) and mid-diestrus (after conceptus implantation), can influence the circulating concentrations of sex steroids, FFA, and vaginal pH in those mice on the VHF diet.

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