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Maternal Fructose and/or Salt Intake and Reproductive Outcome in the Rat: Effects on Growth, Fertility, Sex Ratio, and Birth Order

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ABSTRACT

Maternal diet can significantly skew the secondary sex ratio away from the expected value of 0.5 (proportion males), but the details of how diet may do this are unclear. Here, we altered dietary levels of salt (4% salt in the feed) and/or fructose (10% in the drinking water) of pregnant rats to model potential effects that consumption of a “Western diet” might have on maternal-fetal growth, development, and sex ratio. We demonstrate that excess fructose consumption before and during pregnancy lead to a marked skew in the secondary sex ratio (proportion of excess fructose consumption before and during pregnancy lead to a marked skew in the secondary sex ratio (proportion of males, 0.60; P < 0.006). The effect was not mediated by selective developmental arrest of female embryos or influenced by fetal position in the uterine horn or sex-specific effects on sperm motility, suggesting a direct effect of glycolyzable monosaccharide on the maternal ovary and/or ovulated oocyte. Furthermore, combined excess maternal consumption of salt and fructose-sweetened beverage significantly reduced fertility, reflected as a 50% reduction in preimplantation and term litter size. In addition, we also noted birth order effects in the rat, with sequential implantation sites tending to be occupied by the same sex.

fertility, fructose, nutrition, rat, reproduction, salt, sex ratio

INTRODUCTION

During syngamy, the union of two haploid sex chromosomes (x and/or y) to form a diploid gamete generally produces, considered at a population level, a 50:50 ratio of males to females. However, studying specific populations produces, considered at a population level, a 50:50 ratio of males and females. However, studying specific populations under different environmental conditions has demonstrated that subtle deviations from this ratio can readily occur; for example, altered maternal hormone concentrations, plane of nutrition and food availability, or exposure to endocrine disrupting chemicals may result in significant shifts in the secondary sex ratio [1, 2]. Previously, Trivers and Willard [3] proposed a hypothesis to potentially explain this phenomena: females in better body condition (i.e., more energy reserves in adipose tissue) would produce a greater proportion of male offspring, as males would benefit more from increased parental investment; would become bigger and stronger and thus be more likely, as adults, to join the upper echelons of breeding males and distribute their (and their parents) genes more widely among the population [3]. This prediction has been experimentally validated in a number of animal models including ruminants (for review see [2]) but also in humans. A systematic review found 26 of 54 human studies reported population sex ratios that supported the Trivers-Willard hypothesis [3], with only one study reporting evidence against and the remainder observing no effect [4]. A similar analysis of experimental studies in mammals found that 312 of 422 studies supported the hypothesis when maternal body condition was factored into the analysis [5]; that is, mothers with higher plasma fatty acid or glucose concentration (reflective of mothers in “better body condition”) tended to produce more males. Such a biological phenomena has also been recapitulated in the laboratory: elevated fatty acids or glucose in culture medium can shift the sex ratio to favor males due to the “toxic” effect of high glucose levels, particularly on female embryos because of their temporary (before x-inactivation) dual expression of x-linked metabolic enzymes such as glucose-6 phosphatase [6, 7].

Over the last 80 years, the macronutrient content of human diets has changed considerably. For much of their evolution, humans consumed a diet high in fiber, potassium, complex carbohydrates, and protein and low in sodium, refined sugars, and energy density, that is, a “Paleolithic” diet that provided a plant:animal energy ratio of 1:1 with the net acid load being alkaline [8, 9]. Analyses of the diets of modern hunter-gatherer populations support these predictions [9, 10]. Since that time, when physiological and metabolic systems were evolving, there has been a gradual transition away from this Paleolithic diet, with the emergence of agriculture (~7–5000 years ago), through the industrial revolution (approximately the last 100 years). The modern “Westernized diet” has rapidly become low in fiber and high in sodium, simple sugar, and energy density [11]. Such a conflict between our thrifty phenotype and our nutritionally dense diet (particularly fructose and salt) has been proposed as the underpinning of the current epidemic of obesity and its pathophysiological sequelae such as type 2 diabetes and hypertension [12–14]. Obesity and hypertension are often characterized, and type 2 diabetes defined, by high plasma glucose. Thus, consumption of a Westernized diet may have an impact upon the intrauterine environment and potentially influence offspring sex ratio, yet few studies have tested this hypothesis in a polytocous laboratory animal model.
Hence, in the present study, we aimed to test the hypothesis that moderate inclusion of dietary salt (as NaCl in food to represent increased consumption of sodium in the Western world) and/or fructose in the drinking water (to represent increased consumption of sugar-sweetened beverages in the Western world) would elevate circulating plasma glucose and lipids in nonpregnant dams, metabolic effects that would persist into pregnancy and have marked impacts on offspring development and sex ratio. A significant shift in the secondary sex ratio (e.g., to favor males) may arise through selective fertilization and/or implantation of male embryos, gender-specific developmental arrest, and/or sex-specific effects on the motility of sperm in the reproductive tract. Thus, in order to address these mechanisms, we measured the secondary sex ratio (i.e., sex ratio at birth) in a large population of experimentally controlled rats in which maternal diets had been carefully controlled before and during pregnancy. In order to examine the potential effects of such diets on the primary sex ratio and/or preferential blastocyst developmental arrest, we harvested and sexed a smaller cohort of preimplantation blastocysts at Day 4 gestation (term, ~Day 21). In order to address the potential effect of high uterine tract glucose on differential sperm motility, we cultured sex-sorted bovine semen in physiologically high (5 mM) or low glucose concentrations and observed the decline in motility with time.

MATERIALS AND METHODS

In Vivo Animal Studies

All procedures involving animals were carried out under license and in accordance with the Home Office Animals (Scientific Procedures) Act 1986 and were approved by the local ethical review committee of the Biomedical Support Unit, University of Nottingham. Eighty virgin Sprague-Dawley female rats (190–210 g; 8–10 weeks of age) were supplied by Harlan Teklad, kept in a controlled environment (20–22°C) and humidity (55%–65%) and subjected to a 12L:12D cycle. Rats were housed individually after acclimatization and were randomly assigned to one of four treatment groups: 1) control diet (CD, n = 20), fed purified standard chow (TD.08164; Teklad Harlan) and tap water; 2) salt diet (SD, n = 20), fed purified standard chow with 4% NaCl added (TD.08162; Teklad Harlan) and tap water; 3) fructose diet (FD, n = 20), fed purified standard chow (TD.08164; Teklad Harlan) and tap water; 4) fructose/salt diet (FSD, n = 20), fed purified standard chow (TD.08164; Teklad Harlan) and tap water with 10% fructose added.

Dietary details are given in Supplemental Table S1 (all supplemental data are available online at www.bioreprod.org). All rats were fed experimental diets ad libitum for at least 28 days before conception and during the mating period. At Day 4 of gestation (prior to implantation in the rat; term, ~21 days) a proportion of dams (n = 6/dietary group) were humanely euthanized (CO₂ asphyxiation), and preimplantation blastocysts were collected as follows: maternal ovaries and uterine horn were excised into a Petri dish containing 2 ml of PBS; embryos were transferred to a clean Petri dish and counted using a dissecting microscope (12.5× magnification; Leica) and aspirated via microcapillary pipette. Isolated blastocysts were transferred to a clean Petri dish containing 2 ml of PBS through the cervix. Blastocysts were identified using a dissecting microscope (12.5× magnification; Leica) and aspirated via microcapillary pipette. Isolated blastocysts were transferred to a clean Petri dish containing 2 ml of PBS and incubated at 37°C for 5 min. Following thawing, semen was diluted 1:1 with fertilization medium (37.5-µl aliquots) with and without 5 mM glucose and incubated for 5 min at 37°C. At this point (time zero) motility was assessed by placing a 10-µl aliquot on a prewarmed (37°C) slide with coverslip. Using the CASA system and a heated (37°C) microscope stage, four of a possible six areas of the slide were chosen at random (by throwing a dice), and sperm motility parameters were recorded until at least 100 valid tracks had been measured. Motility parameters of interest included percentage of motility, linear movement, and velocity (µm/sec). Motility was assessed at hourly intervals thereafter for up to 5 h, with 3–4 replicates measured at each time point.

Statistics

Briefly, all animal studies were designed with a 2 × 2 factorial structure and were analyzed by mixed effect models, including the dam as a random term to account for reduced intradam variance (Genstat version 14 software; VSNi). All data were first checked for normality (or otherwise) of the residual error distribution and log₁₀-transformed where appropriate. In some instances, data were analyzed with an appropriate adjustment (e.g., effects on birth weight were analyzed with adjustment for litter size). All data are presented as predicted means ± SEM or standard error of the mean (SEM). Differences between treatments were assessed using a chi-square test or Fisher’s exact test (two-tailed) as appropriate. Differences, when both salt and fructose were consumed (Supplemental Fig. S2), by computer-assisted sperm analysis (CASA; sperm tracker 7; Hobson).

RESULTS

Consumption of the Westernized Diet, High in Fructose and/or Salt, Has Little Effect on Weight Gain in Nonpregnant and Pregnant Rats

A purified diet was designed in which the macronutrient content and energy density were identical, with differences only in mineral contents (i.e., increased inclusion of salt, as NaCl [Supplemental Table S1]) to avoid confounding with other aspects of the diet that might differ among treatment groups [15]. In weight-matched (186 ± 3 g) nonpregnant rats, feeding on the diets for 28 days alongside either water or water supplemented with 10% fructose had little effect on weight gain until Day 10 when FSD rats began to gain less weight (Supplemental Fig. S1a). Food intake was unaffected by salt in the diet but was reduced when the fructose-enriched beverage was available (Supplemental Fig. S1b), while fluid intake was significantly increased in each treatment group and exacerbated when both salt and fructose were consumed (Supplemental Fig. S1c). Taken together, energy intake in the nonpregnant dam was similar to those of control and salt-fed rats but marginally increased in rats consuming fructose (55.8 ± 0.7 vs. 51.0 ± 0.8 kcal/day [Supplemental Fig. S1d]). After 4–5 weeks and an average weight of 238 ± 3 g, rats were mated and fed the diets during gestation. Weight was gained at a similar rate on each diet until Day 15 when FSD rats, again, tended to gain less weight (Supplemental Fig. S1e), partially because of reduced average litter size in this group (7 pups vs. 14 pups for all other litters in this group (7 pups vs. 14 pups for all other litters in this group).
groups). While SD rats drank more fluid (Supplemental Fig. S1g), fructose-fed rats ate less and drank more fluid (Supplemental Fig. S1, f and g), on average consuming more daily calories (13.0±6.6 kcal/day [Supplemental Fig. S1h]).

Pregnancy and Consumption of a Westernized Diet Combine To Influence Maternal Plasma Biochemistry

In the rat, being pregnant significantly increased plasma TAG (Fig. 1) and uric acid (Supplemental Table S3) per se, regardless of diet. Consuming a fructose-sweetened beverage increased maternal plasma cholesterol (2.7 ± 0.14 vs. 2.3 ± 0.14 mmol/L; P = 0.05), glucose, and TAG (Fig. 1, A–D), but maternal plasma insulin was unaltered by diet (pooled estimate, 1.32 ± 0.04 ng/ml). Maternal salt intake (adjusted for water intake) significantly increased urine production rate (27.2 ± 5.4 vs. 7.3 ± 2.3 μl/min; P = <0.001) and urinary albumin:creatinine ratio (1.19 vs. 0.82 ± 0.13 g/μmol/L; P = 0.01), indicating microalbuminuria and decreased renal barrier function. Toward the end of gestation, maternal weight was on average 13 ± 4 g less in fructose-fed animals, largely because of the reduced weight (adjusted for litter size) in the FSD group (343 ± 4.0 g for the FSD group vs. 364 ± 4.7 g for the CD group).

Consumption of a Westernized Diet Has Little Effect on Weight Gain in Nonpregnant and Pregnant Rats But Has Marked Effects on Fetal Growth and Secondary Sex Ratio

In contrast to the dam, fetal plasma glucose and fructose at Day 20 (0.95 gestation) were unaffected by excess maternal fructose intake (Supplemental Table S4). At birth, male fetuses were heavier than female fetuses, but fructose-exposed female offspring were significantly smaller than control siblings (Fig. 1A and C).
Fructose-exposed male offspring appeared to be influenced by maternal diet, but the effect did not reach statistical significance. Analysis of the increase in weight from Day 20 to term (from cross-sectional data obtained at term [21 ± 1 days] and at Day 20 gestation) illustrated the remarkable growth of rat fetuses over the previous 24–36 h (~5%) of gestation, with females and males increasing weight by 88% ± 1% and 85% ± 1%, respectively (Fig. 2B), and with those exposed to fructose or salt diet alone exhibiting a degree of growth restriction (Fig. 2B). Maternal consumption of fructose and salt (i.e., FSD diet) elicited considerable reproductive effects: litter size was reduced at term (Fig. 2C) and prior to implantation at Day 4 gestation (7 [7–8.5] vs. 12 [10–12] blastocysts; FSD vs. all other groups; median [interquartile range], P = 0.004), and birth weight and late gestation fetal growth were also rescued (Fig. 2, A and B). Furthermore, we observed three stillbirths in the FSD group but none in any other group. Modeling of binomial proportions (in a total of 631 offspring; 347 males, 284 females) indicated a significant shift toward more males in the litter after maternal consumption of fructose (61% ± 1.9% vs. 48% ± 2.2% males; F_{stat} = 7.9, F_{prob} = 0.005) (Fig. 2D). Maternal diet had no effect on the position of offspring within the uterus, and uterine position itself did not impact offspring birth weight at all. However, the sequence of implanted fetuses within uterine horns was not random: we observed a significant difference in the relative frequencies of males and females within the three categories of uterine position (Table 1). A male or female fetus was more likely to be surrounded by a pair of fetuses of its own sex than by a pair of the other sex.

FIG. 2. Excess intake of fructose-sweetened beverage reduces fetal growth and significantly skews the offspring sex ratio to favor males, regardless of litter size. Birth weight (A), late fetal growth (B), litter size (C), and sex ratio (D) of rat pups from litters of dams fed 1) control diet and water ad libitum (n = 6); 2) control diet and 10% fructose in water ad libitum (n = 6); 3) 4% salt diet and water ad libitum (n = 6); and 4) 4% salt diet and 10% fructose in water ad libitum (n = 6). B) Cross-sectional weight data from two different cohorts of dams euthanized either at Day 20 or at term after consuming the diets as described in Materials and Methods. The mean percent increase in weight was calculated from the averages of each dietary group at each time point (i.e., Day 20 and term [~Day 21 in the rat]). C) Data points are individual litter sizes from dietary groups determined at Day 20 or at term. D) Sex ratio was calculated as the proportion of males (number of males/total number in litter) and tested against the expected proportion of 0.50. Data were analyzed using general linear mixed model (Genstat version 14 software). *P < 0.05 for an effect of fructose; †P < 0.05 for a significant interaction of sex with fructose and salt.
TABLE 1. Birth order has an influence on fetal gender.

<table>
<thead>
<tr>
<th>Count</th>
<th>Male/male</th>
<th>Female/female</th>
<th>Male/female</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>55</td>
<td>15</td>
<td>61</td>
</tr>
<tr>
<td>Females</td>
<td>16</td>
<td>35</td>
<td>59</td>
</tr>
</tbody>
</table>

* We observed significant differences (chi-square test $\chi^2 = 27.8; P < 0.001$) among the relative frequencies of males and those of females within the three categories.

Excess Glucose Has Little Effect on Sperm Motility In Vitro

Sex-sorted spermatozoa were significantly less active (Fig. 3, A and B) than unsorted mixed spermatozoa after freeze-thawing. In addition, the decline in these parameters over time was similar between mixed and sex-sorted spermatozoa. Incubation with glucose had little effect on any of these parameters, but the percentage spermatozoa designated as motile was higher in sex-sorted than in mixed spermatozoa (Fig. 3E).

DISCUSSION

We demonstrate for the first time a clear increase in the proportion of males born at term after feeding dams a diet high in fructose, and a marked impact on fertility (number of offspring at term) when combined with a diet high in salt. In general, these results support the sex allocation hypothesis originally proposed by Trivers and Willard [3]; that is, females in better body condition with the greatest food resource (e.g., marked by higher plasma fatty acid and glucose concentrations) produce more male than female offspring. Furthermore, we also provide evidence that the shift in sex ratio likely has its origin in the ovary and subsequently ovulated oocyte, as we saw 1) no greater selective developmental arrest of female embryos (the male:female ratios were similar prior to implantation and at term and in large and small litters); 2) no effect of intrauterine position (i.e., secondary sex ratio was not affected by any given offspring being surrounded by two males, two females, or a male/female); and 3) no effect of high glucose on the motility of sex-sorted sperm. We also observed that the sequence of offspring implanted within the uterine horns was not random but appeared significantly influenced by gender; that is, fetuses were more likely to implant in apparent “runs” of the same sex.

A modern, Westernized diet is high in sodium and simple sugars [11]. Few studies have considered the potential effects that consumption of such a diet may have on reproductive function. We specifically chose to add increased fructose and salt to the drinking water and diet, respectively, to represent the temporal change in the consumption of simple sugars (especially fructose) and salt (i.e., from sugar-sweetened beverages and increasingly refined diets, respectively) that Westernized societies have witnessed over the past 20 years. Furthermore, while all cells of the body are able to metabolize excess glucose, the liver is the obligatory site for metabolism of fructose; hence, excess intake of fructose (but not glucose) is associated with many metabolic diseases [12]. The levels of intake of these nutrients in our experimental model (salt, 15-fold increase above requirements; fructose, 15%–25% calorific intake) are moderately high for a rat, but much greater levels of incorporation have been used previously (e.g., 8% salt intake, 66% fructose intake). Furthermore, the relative intake is comparable to, for example, the quantities consumed by pregnant women living in a Westernized society. For example, historically, it is estimated that Homo sapiens likely evolved consuming a diet with $\approx 250$ mg/day salt, deriving no more than 2% energy from simple sugars [9, 16]. The current estimated average consumption of $\approx 8$–12 g/day salt and 18%–25% of energy from simple sugars represents a 32- to 48-fold increase in salt and 9- to 12-fold increase in simple sugar intake, respectively. In addition, using purified diets allows better clarification of causative main effects (such as salt or fructose) in the diet and is free from confounding influences that limit the interpretation of many other nutritional studies in laboratory species [15]. While rats consuming a fructose-sweetened beverage had a reduction in food and, thus, vitamin and mineral intake, it is highly unlikely these rats were not nutritionally replete given the small reduction in their intake of food (12%–15%) and considering the level of incorporation in the diets relative to a rodents’ micronutrient requirement during gestation. In our study, excess salt or fructose intake in isolation had predictable effects on the dams such as eliciting polyuria and polydipsia with little effect on weight gain during pregnancy or other measures of reproductive success (e.g., litter size and birth weight). However, when consumed together, clear effects on reproductive success (i.e., fertility) of dams were noted: measured litter size was reduced at term and prior to implantation in the rat, and observational data also indicated that dams consuming both salt and fructose had difficulty becoming pregnant relative to all other groups. In the spiny mouse, increased diet salinity (from 2.5% to 5% inclusion) led to a reproductive hiatus, and the authors speculate that such an effect, mediated through increased vasopressin, acted as an ecological strategy to signal water shortage and the end of the seasonal reproductive period in a hot desert environment [17]. Reproduction, alongside growth and maintenance, competes for energy in the context of a concerted life history strategy [18, 19] and is obviously well protected but nevertheless sensitive to nutritional challenges. For example, after 12 generations of protein-energy malnutrition in the rat, adult body weight and litter size were marginally reduced but there was little impact on fecundity per se [20], illustrating a tradeoff of energy away from anabolic processes toward maintenance of reproductive function, as observed in pigmies [18]. For a polytocous species such as the rat, (i.e. one in which many live offspring are procured in a single birth), then, the tradeoff is relatively easy: fertility may be reduced by a lower ovulation rate. Data presented here also suggest, but do not confirm, a lowering of ovulation rate in the rat after consumption of excess salt and fructose, as we did not witness any evidence of failed implantation sites and/or increased reabsorption of embryos. The extent to which these data and conclusions can be extended to monovular species is uncertain; certainly in humans and cows there is robust evidence that malnutrition (both under- and overnutrition) can influence the reproductive axis and affect fertility in the short term (for a review see [21]) but that in the long term, lifetime fecundity is rarely affected [22].

In the present study, dams offered fructose as part of their dietary regimen tended to consume more energy, and their plasma biochemistry test results were reflective of a dam, according to Trivers and Willard [3], in better body condition; that is, increased maternal fatty acids, TAG, and glucose potentially mark greater energy availability for the fetus. Such effects are commonly observed when fructose is consumed in excess due to the reliance and dependence of fructose metabolism on the liver, de novo hepatic lipogenesis, and glucose output increase [12]. While maternal diet, particularly a calorie excess, has previously been shown to directly influence sex ratio, with more males than females being born under such
FIG. 3. Excess glucose has little effect on sperm motility in vitro. Spermatozoa motility was assessed in three to four replicates per time point on n = five to six separate occasions (using different frozen straws). CASA was used to determine the motility parameters; percentage of active sperm (A and B), linear velocity (C), mean path velocity (D), and percentage of motile sperm (E). Data are means predicted from the model, with overall SED used to represent the error. Data were analyzed by mixed effects models with treatment (with or without glucose) and time or their interaction as fixed effects and spermatozoa batch as a random effect.
conditions [2, 23], we show here that a milder phenotype and specifically that fructose intake per se markedly shifts the sex ratio in favor of males. We suggest, however, that our data are likely explained by the secondary effect of high maternal glucose concentration, as experimental manipulation of the plasma glucose concentration around the time of conception influences the litter sex ratio in a mammal [24] and in vitro studies using the mouse embryo cultured in the presence of high glucose, but not fructose, similarly lead to a greater proportion of male blastocysts surviving [6]. Furthermore, we clarify in vivo that the effect is likely at the level of the ovary and/or preimplantation blastocyst and unlikely to be a paternal effect as breeding males in our study only received experimental diet during the mating period (average, 3 ± 1 days), much less than the duration of rodent spermatogenesis. Moreover, while differential motility of x- or y-bearing spermatozoa within the reproductive tract remains a possible explanation, we present evidence to suggest it is unlikely; sex-sorted spermatozoa had reduced parameters of motility relative to those in unsorted semen, as observed previously [25], but there appeared to be no overall effect of high-glucose concentration in the fertilization medium on spermatozoal motility.

Finally, as previously speculated, we find no evidence that the relative proportion of male or female fetuses within a litter significantly influences their birth weight; weights in polytocous species are largely influenced by litter size and fetal sex per se rather than by gender distribution. A similar lack of effect has been observed by the authors of studies in sheep, a species that usually gives birth to between one and three offspring [26]. However, it is of interest and we report for the first time in a polytocous species, to the best of our knowledge, the observation of a significant bias toward fetuses implanting in runs of the same sex, whether male or female, and that this effect was not influenced by aspects of our dietary design (Table 1). Such a phenomenon has been observed previously in the monotocous human that if the first-born is a boy, then the likelihood of having another boy is increased [27]. To therefore show in a polytocous species that the likelihood of a male or female fetus implanting appears to be influenced by the sex of their neighboring fetuses, adds support to the biologic validity of birth order effects in mammals. Our study, along with the previous observation [27], offers no potential mechanistic insight, and we can only echo the previous authors’ conclusions; it remains an enigma, but we speculate that the placental microenvironment upon implantation of a male or female fetus is locally altered to favor implantation of another fetus of the same sex. In a number of species with multiple offspring, should placental arteriovenous anastomoses develop between fetuses in a mixed-sex pregnancy (“freemartinism,” i.e., the exchange of cells and hormones between fetuses), then the female is often rendered sterile, because of the presence of male hormones [28]. Perhaps an evolutionary pressure encourages implantation of successive same-sex fetuses to mitigate against such biological consequences of chimerism.

To conclude, shifting of the sex ratio by maternal diet in mammals has been demonstrated. This study adds to the current literature in witnessing this phenomenon but extends it in isolating a direct role for changes in maternal fructose intake (and likely secondary effect of increased maternal plasma glucose) acting early in gestation. While a number of other potential explanations can be dismissed, for example, sex-specific effects on spermatozoa or selective loss of female embryos, we cannot exclude other nutritional factors that are confounded by a high-fructose diet, such as increased plasma nonesterified fatty acids, TAG, and cholesterol. We speculate, however, that maternal diet prior to conception is an important factor with regard to influencing the sex of the fetus. A mother in good body condition with higher than average glucose, fatty acids, and TAG is more likely, in polytocous species at least, to have male rather than female offspring. The molecular mechanism of such a developmental cue likely resides in the oocyte and/or its sensitivity to hormonal or nutritional cues from the local microenvironment.

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